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(54) Title: REGULATION OF EPITHELIAL TISSUE BY HEDGEHOG-LIKE POLYPEPTIDES, AND FORMULATIONS AND USES RELATED THERETO

(57) Abstract

The present application relates to a method for modulating the growth state of an epithelial cell by ectopically contacting the epithelial cell, *in vitro* or *in vivo*, with a hedgehog therapeutic or ptc therapeutic in an amount effective to alter the rate (promote or inhibit) of proliferation of the epithelial cell, e.g., relative to the absence of administration of the hedgehog therapeutic or ptc therapeutic. The subject method can be used, for example, to modulate the growth state of an epithelial tissue, such as for inducing the formation of skin or other cutaneous tissue, or for inducing growth of hair.

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Regulation of Epithelial Tissue by Hedgehog-like Polypeptides, and Formulations and Uses Related Thereto

Background of the Invention

Pattern formation is the activity by which embryonic cells form ordered spatial arrangements of differentiated tissues. The physical complexity of higher organisms arises during embryogenesis through the interplay of cell-intrinsic lineage and cell-extrinsic signaling. Inductive interactions are essential to embryonic patterning in vertebrate development from the earliest establishment of the body plan, to the patterning of the organ systems, to the generation of diverse cell types during tissue differentiation (Davidson, E., (1990) *Development* 108: 365-389; Gurdon, J. B., (1992) *Cell* 68: 185-199; Jessell, T. M. et al., (1992) *Cell* 68: 257-270). The effects of developmental cell interactions are varied. Typically, responding cells are diverted from one route of cell differentiation to another by inducing cells that differ from both the uninduced and induced states of the responding cells (inductions). Sometimes cells induce their neighbors to differentiate like themselves (homoiogenetic induction); in other cases a cell inhibits its neighbors from differentiating like itself. Cell interactions in early development may be sequential, such that an initial induction between two cell types leads to a progressive amplification of diversity. Moreover, inductive interactions occur not only in embryos, but in adult cells as well, and can act to establish and maintain morphogenetic patterns as well as induce differentiation (J.B. Gurdon (1992) *Cell* 68:185-199).

Members of the *Hedgehog* family of signaling molecules mediate many important short- and long-range patterning processes during invertebrate and vertebrate development. In the fly a single *hedgehog* gene regulates segmental and imaginal disc patterning. In contrast, in vertebrates a *hedgehog* gene family is involved in the control of left-right asymmetry, polarity in the CNS, somites and limb, organogenesis, chondrogenesis and spermatogenesis.

The first *hedgehog* gene was identified by a genetic screen in the fruitfly *Drosophila melanogaster* (Nüsslein-Volhard, C. and Wieschaus, E. (1980) *Nature* 287, 795-801). This screen identified a number of mutations affecting embryonic and larval development. In 1992 and 1993, the molecular nature of the *Drosophila hedgehog* (*hh*) gene was reported (*C.F.*, Lee et al. (1992) *Cell* 71, 33-50), and since then, several *hedgehog* homologues have been isolated

from various vertebrate species. While only one *hedgehog* gene has been found in *Drosophila* and other invertebrates, multiple *Hedgehog* genes are present in vertebrates.

The various Hedgehog proteins consist of a signal peptide, a highly conserved N-terminal region, and a more divergent C-terminal domain. In addition to signal sequence cleavage in the secretory pathway (Lee, J.J. *et al.* (1992) *Cell* 71:33-50; Tabata, T. *et al.* (1992) *Genes Dev.* 2635-2645; Chang, D.E. *et al.* (1994) *Development* 120:3339-3353), Hedgehog precursor proteins undergo an internal autoproteolytic cleavage which depends on conserved sequences in the C-terminal portion (Lee *et al.* (1994) *Science* 266:1528-1537; Porter *et al.* (1995) *Nature* 374:363-366). This autocleavage leads to a 19 kD N-terminal peptide and a C-terminal peptide of 26-28 kD (Lee *et al.* (1992) *supra*; Tabata *et al.* (1992) *supra*; Chang *et al.* (1994) *supra;* Lee *et al.* (1994) *supra*; Bumcrot, D.A., *et al.* (1995) *Mol. Cell. Biol.* 15:2294-2303; Porter *et al.* (1995) *supra*; Ekker, S.C. *et al.* (1995) *Curr. Biol.* 5:944-955; Lai, C.J. *et al.* (1995) *Development* 121:2349-2360). The N-terminal peptide stays tightly associated with the surface of cells in which it was synthesized, while the C-terminal peptide is freely diffusible both *in vitro* and *in vivo* (Lee *et al.* (1994) *supra*; Bumcrot *et al.* (1995) *supra;* Mart', E. *et al.* (1995) *Development* 121:2537-2547; Roelink, H. *et al.* (1995) *Cell* 81:445-455). Interestingly, cell surface retention of the N-terminal peptide is dependent on autocleavage, as a truncated form of HH encoded by an RNA which terminates precisely at the normal position of internal cleavage is diffusible *in vitro* (Porter *et al.* (1995) *supra*) and *in vivo* (Porter, J.A. *et al.* (1996) *Cell* 86, 21-34). Biochemical studies have shown that the autoproteolytic cleavage of the HH precursor protein proceeds through an internal thioester intermediate which subsequently is cleaved in a nucleophilic substitution. It is likely that the nucleophile is a small lipophilic molecule which becomes covalently bound to the C-terminal end of the N-peptide (Porter *et al.* (1996) *supra*), tethering it to the cell surface. The biological implications are profound. As a result of the tethering, a high local concentration of N-terminal Hedgehog peptide is generated on the surface of the Hedgehog producing cells. It is this N-terminal peptide which is both necessary and sufficient for short and long range Hedgehog signaling activities in *Drosophila* and vertebrates (Porter *et al.* (1995) *supra;* Ekker *et al.* (1995) *supra;* Lai *et al.* (1995) *supra*; Roelink, H. *et al.* (1995) *Cell* 81:445-455; Porter *et al.* (1996) *supra;* Fietz, M.J. *et al.* (1995) *Curr. Biol.* 5:643-651; Fan, C.-M. *et al.* (1995) *Cell* 81:457-465; Mart', E., *et al.* (1995) *Nature* 375:322-325; Lopez-Martinez *et al.* (1995) *Curr.*

Biol 5:791-795; Ekker, S.C. *et al.* (1995) *Development* 121:2337-2347; Forbes, A.J. *et al.*(1996) *Development* 122:1125-1135).

HH has been implicated in short- and longe range patterning processes at various sites during *Drosophila* development. In the establishment of segment polarity in early embryos, it has short range effects which appear to be directly mediated, while in the patterning of the imaginal discs, it induces long range effects via the induction of secondary signals.

In vertebrates, several *hedgehog* genes have been cloned in the past few years (see Table 1). Of these genes, *Shh* has received most of the experimental attention, as it is expressed in different organizing centers which are the sources of signals that pattern neighbouring tissues. Recent evidence indicates that *Shh* is involved in these interactions.

The interaction of a hedgehog protein with one of its cognate receptor, *patched*, sets in motion a cascade involving the activation and inhibition of downstream effectors, the ultimate consequence of which is, in some instances, a detectable change in the transcription or translation of a gene. Transcriptional targets of *hedgehog* signaling are the *patched* gene itself (Hidalgo and Ingham, 1990 *Development* 110, 291-301; Marigo et al., 1996) and the vertebrate homologs of the drosophila cubitus interruptus (Ci) gene, the *GLI* genes (Hui et al. (1994) *Dev Biol* 162:402-413). *Patched* gene expression has been shown to be induced in cells of the limb bud and the neural plate that are responsive to *Shh*. (Marigo et al. (1996) *Development* 122:1225-1233). The *GLI* genes encode putative transcription factors having zinc finger DNA binding domains (Orenic et al. (1990) *Genes & Dev* 4:1053-1067; Kinzler et al. (1990) *Mol Cell Biol* 10:634-642). Transcription of the *GLI* gene has been reported to be upregulated in response to *hedgehog* in limb buds, while transcription of the *GLI3* gene is downregulated in response to *hedgehog* induction (Marigo et al. (1996) *Development* 122:1225-1233). Moreover, it has been demonstrated that elevated levels of Ci are sufficient to activate *patched* (ptc) and other *hedgehog* target genes, even in the absence of *hedgehog* activity.

Summary of the Invention

One aspect of the present application relates to a method for modulating the growth state of an epithelial cell by ectopically contacting the epithelial cell, *in vitro* or *in vivo*, with a hedgehog therapeutic or ptc therapeutic in an amount effective to alter the rate (promote or

inhibit) of proliferation of the epithelial cell, e.g., relative to the absence of administeration of the hedgehog therapeutic or ptc therapeutic. The subject method can be used, for example, to modulate the growth state of an epithelial tissue, such as for inducing the formation of skin or other cutaneous tissue, or for inducing growth of hair.

Wherein the subject method is carried out using a *hedgehog* therapeutic, the *hedgehog* therapeutic preferably a polypeptide including a *hedgehog* portion comprising at least a bioactive extracellular portion of a *hedgehog* protein, e.g., the *hedgehog* portion includes at least 50, 100 or 150 (contiguous) amino acid residues of an N-terminal half of a *hedgehog* protein.In preferred embodiments, the *hedgehog* portion includes at least a portion of the *hedgehog* protein corresponding to a 19kd fragment of the extracellular domain of a *hedgehog* protein.

In certain preferred embodiments, the *hedgehog* portion has an amino acid sequence at least 60, 75, 85, or 95 percent identical with a hedgehog protein of any of SEQ ID Nos. 10-18 or 20, though sequences identical to those sequence listing entries are also contemplated as useful in the present method. The *hedgehog* portion can be encoded by a nucleic acid which hybridizes under stringent conditions to a nucleic acid sequence of any of SEQ ID Nos. 1-9 or 19, e.g., the *hedgehog* portion can be encoded by a vertebrate *hedgehog* gene, especially a human *hedgehog* gene.

In certain embodiments, the hedgehog polypeptide is modified with one or more sterol moieties, e.g., cholesterol or a derivative thereof.

In certain embodiments, the hedgehog polypeptide is modified with one or more fatty acid moieties, such as a fatty acid moiety selected from the group consisting of myristoyl, palmitoyl, stearoyl, and arachidoyl.

In certain embodiments, the hedgehog polypeptide is modified with one or more aromatic hydrocarbons, such as benzene, perylene, phenanthrene, anthracene, naphthalene, pyrene, chrysene, or naphthacene.

In certain embodiments, the hedgehog polypeptide is modified one or more times with a C7 - C30 alkyl or cycloalkyl.

In other embodiments, the subject method can be carried out by administering a gene activation construct, wherein the gene activation construct is deigned to recombine with a genomic *hedgehog* gene of the patient to provide a heterologous transcriptional regulatory sequence operatively linked to a coding sequence of the *hedgehog* gene.

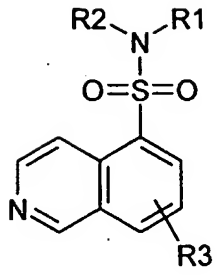
In still other embodiments, the subject method can be practiced with the administration of a gene therapy construct encoding a *hedgehog* polypeptide. For instance, the gene therapy construct can be provided in a composition selected from a group consisting of a recombinant viral particle, a liposome, and a poly-cationic nucleic acid binding agent,

In yet other embodiments, the subject method can be carried out using a ptc therapeutic. An exemplary ptc therapeutic is a small organic molecule which binds to a *patched* protein and derepresses *patched*-mediated inhibition of mitosis, e.g., a molecule which binds to *patched* and mimics *hedgehog*-mediated *patched* signal transduction, which binds to *patched* and regulates *patched*-dependent gene expression. For instance, the binding of the ptc therapeutic to *patched* may result in upregulation of patched and/or gli expression.

In a more generic sense, the *ptc* therapeutic can be a small organic molecule which interacts with epithelial cells to induce *hedgehog*-mediated *patched* signal transduction, such as by altering the localization, protein-protein binding and/or enzymatic activity of an intracellular protein involved in a *patched* signal pathway. For instance, the *ptc* therapeutic may alter the level of expression of a *hedgehog* protein, a patched protein or a protein involved in the intracellular signal transduction pathway of *patched*.

In certain embodiments, the *ptc* therapeutic is an antisense construct which inhibits the expression of a protein which is involved in the signal transduction pathway of *patched* and the expression of which antagonizes *hedgehog*-mediated signals. The antisense construct is perferably an oligonucleotide of about 20-30 nucleotides in length and having a GC content of at least 50 percent.

In other embodiments, the *ptc* therapeutic is an inhibitor of protein kinase A (PKA), such as a 5-isoquinolinesulfonamide. The PKA inhibitor can be a cyclic AMP analog. Exemplary PKA inhibitors include N-[2-((p-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide, 1-(5-isoquinoline-sulfonyl)-2-methylpiperazine, KT5720, 8-bromo-



wherein,

R_1 and R_2 each can independently represent hydrogen, and as valence and stability permit a lower alkyl, a lower alkenyl, a lower alkynyl, a carbonyl (such as a carboxyl, an ester, a formate, or a ketone), a thiocarbonyl (such as a thioester, a thioacetate, or a thioformate), an amino, an acylamino, an amido, a cyano, a nitro, an azido, a sulfate, a sulfonate, a sulfonamido, $-(CH_2)_m-R_8$, $-(CH_2)_m-OH$, $-(CH_2)_m-O$-lower alkyl, $-(CH_2)_m-O$-lower alkenyl, $-(CH_2)_n-O-(CH_2)_m-R_8$, $-(CH_2)_m-SH$, $-(CH_2)_m-S$-lower alkyl, $-(CH_2)_m-S$-lower alkenyl, $-(CH_2)_n-S-(CH_2)_m-R_8$, or

R_1 and R_2 taken together with N form a heterocycle (substituted or unsubstituted);

R_3 is absent or represents one or more substitutions to the isoquinoline ring such as a lower alkyl, a lower alkenyl, a lower alkynyl, a carbonyl (such as a carboxyl, an ester, a formate, or a ketone), a thiocarbonyl (such as a thioester, a thioacetate, or a thioformate), an amino, an acylamino, an amido, a cyano, a nitro, an azido, a sulfate, a sulfonate, a sulfonamido, $-(CH_2)_m-R_8$, $-(CH_2)_m-OH$, $-(CH_2)_m-O$-lower alkyl, $-(CH_2)_m-O$-lower alkenyl, $-(CH_2)_n-O-(CH_2)_m-R_8$, $-(CH_2)_m-SH$, $-(CH_2)_m-S$-lower alkyl, $-(CH_2)_m-S$-lower alkenyl, $-(CH_2)_n-S-(CH_2)_m-R_8$;

R_8 represents a substituted or unsubstituted aryl, aralkyl, cycloalkyl, cycloalkenyl, or heterocycle; and

n and m are independently for each occurrence zero or an integer in the range of 1 to 6.

The subject method can be used to treat, e.g., a epithelial disorder, such as in the control of a wound healing process. For instance, the subect method can be used as part of such treatments as burn treatment, skin regeneration, skin grafting, pressure sore treatment,

dermal ulcer treatment, post surgery scar reduction and treatment of ulcerative colitis. In the control of hair growth, the subject method can used as part of a treatment of alopecia.

Yet another aspect of the present invention concerns preparations of a *hedgehog* or *ptc* therapeutic formulated for topical application to epithelial tissue, e.g., to skin. For example, such formulations may include a polypeptide comprising a hedgehog polypeptide sequence including a bioactive fragment of a *hedgehog* protein, which polypeptide is formulated for topical application to epithelial tissue.

Detailed Description of the Invention

Figures 1A, B and C illustrate the induction of hair growth on mice treated with various hedgehog formulations.

Detailed Description of the Invention

Normal skin epidermis is a complex epithelial tissue containing keratinocytes that are proliferating, differentiating and desquamating, and is stratified such that morphological and functional changes in the keratinocytes occur in an orderly progression. The normal epidermis is maintained in a dynamic steady state as proliferation of keratinocytes continually compensates for the loss of cells which are shed from the surface of the skin. Within the epidermis, proliferation takes place in the basal layer of keratinocytes that are attached to the underlying basement membrane, and cells undergo terminal differentiation as they migrate through the suprabasal layers, finally being shed from the tissue surface as dead, cornified squames. Three subpopulations of basal keratinocytes have been defined by cell kinetic analysis: stem cells, transit-amplifying cells, and committed cells. Stem cells retain a high capacity for self-renewal throughout adult life and are ultimately responsible for epidermal maintenance and repair. The progeny of stem cells can either be stem cells themselves or cells known as transit-amplifying cells. Transit-amplifying cells divide a small number of times, but have a high probability of producing daughters that withdraw irreversibly from the cell cycle and are committed to differentiate terminally.

I. Overview

The present application is directed to the discovery that preparations of hedgehog polypeptides can be used to control the formation and/or maintenance of epithelial tissue. As described in the appended examples, *hedgehog* proteins are implicated in the proliferation of epithelial stem cells and may provide early signals that regulate the differentiation of the stem cells into epithelial tissues. In general, the method of the present invention comprises contacting an epithelial cell with an amount of a hedgehog therapeutic (defined infra) which produces a non-toxic response by the cell of (i) induction of epithelial tissue formation or (ii) inhibition of epithelial tissue formation, depending on the whether the hedgehog therapeutic is a sufficient hedgehog agonist or hedgehog antagonist. The subject method can be carried out on epithelial cells which may be either dispersed in culture or a part of an intact tissue or organ. Moreover, the method can be performed on cells which are provided in culture (*in vitro*), or on cells in a whole animal (*in vivo*).

In one aspect, the present invention provides pharmaceutical preparations and methods for controlling the proliferation of epithelially-derived tissue utilizing, as an active ingredient, a *hedgehog* polypeptide or a mimetic thereof. The invention also relates to methods of controlling proliferation of epithelial-derived tissue by use of the pharmaceutical preparations of the invention.

The *hedgehog* formulations of the present invention may be used as part of regimens in the treatment of disorders of, or surgical or cosmetic repair of, such epithelial tissues as skin and skin organs; corneal, lens and other ocular tissue; mucosal membranes; and periodontal epithelium. The methods and compositions disclosed herein provide for the treatment or prevention of a variety of damaged epithelial and mucosal tissues. For instance, the subject method can be used to control wound healing processes, as for example may be desirable in connection with any surgery involving epithelial tissue, such as from dermatological or periodontal surgeries. Exemplary surgical repair for which *hedgehog* therapy is a candidate treatment include severe burn and skin regeneration, skin grafts, pressure sores, dermal ulcers, fissures, post surgery scar reduction, and ulcerative colitis.

In another aspect of the present invention, *hedgehog* preparations can be used to effect the growth of hair, as for example in the treatment of alopecia whereby hair growth is

potentiated, or for example in cosemetic removal of hair (depilation) whereby hair growth is inhibited.

In certain embodiments, the subject compositions can be used to inhibit, rather than promote, growth of epithelial-derived tissue. For instance, certain of the compositions disclosed herein may be applied to the treatment or prevention of a variety hyperplastic or neoplastic conditions. The method can find application for the treatment or prophylaxis of, e.g., psoriasis; keratosis; acne; comedogenic lesions; folliculitis and pseudofolliculitis; keratoacanthoma; callosities; Darier's disease; ichthyosis; lichen planus; molluscous contagiosum; melasma; Fordyce disease; and keloids or hypertrophic scars. Certain of the formulations of the present invention may also be used as part of treatment regimens in auto-immune diseases for affecting healing of proliferative manifestations of the disorder, as for example, part of a treatment for aphthous ulcers, pemphigus such as pemphigus vulgaris, pemphigus foliaceus, pemphigus vegetans or pemphigus erythematous, epidermolysis, lupus lesions or desquamative lesions.

The subject *hedgehog* treatments are effective on both human and animal subjects afflicted with these conditions. Animal subjects to which the invention is applicable extend to both domestic animals and livestock, raised either as pets or for commercial purposes. Examples are dogs, cats, cattle, horses, sheep, hogs and goats.

Still another aspect of the present invention provides a method of stimulating the growth and regulating the differentiation of epithelial tissue in tissue culture.

Without wishing to be bound by any particular theory, the induction of stem cell proliferation by hedgehog proteins may be due at least in part to the ability of these proteins to antagonize (directly or indirectly) *patched*-mediated regulation of gene expression and other physiological effects mediated by that protein. The *patched* gene product, a cell surface protein, is understood to signal through a pathway which causes transcriptional repression of members of the Wnt and Dpp/BMP families of morphogens, proteins which impart positional information. In development of the CNS and patterning of limbs in vertebrates, the introduction of *hedgehog* relieves (derepresses) this inhibition conferred by *patched*, allowing expression of particular gene programs.

Recently, it has been reported that mutations in the human version of *patched*, a gene first identified in a fruit fly developmental pathway, cause a hereditary skin cancer and may contribute to sporadic skin cancers. See, for example, Hahn et al. (1996) *Cell* 86:841-851; and Johnson et al. (1996) *Science* 272:1668-1671. The demonstraction that nevoid basal-cell carcinoma (NBCC) results from mutations in the human *patched* gene provided an example of the roles *patched* plays in post-embryonic deveolpment. These observations have led the art to understand one activity of *patched* to be a tumor suppressor gene, which may act by inhibiting proliferative signals from *hedgehog*. Our observations set forth below reveal potential new roles for the *hedgehog/patched* pathway in maintenance of epithelial cell proliferation and differentiation. Accordingly, the present invention contemplates the use of other agents which are capable of mimicking the effect of the *hedgehog* protein on *patched* signalling, e.g., as may be identified from the drug screening assays described below.

II. Definitions

For convience, certain terms employed in the specfication, examples, and appended claims are collected here.

The term "hedgehog therapeutic" refers to various forms of hedgehog polypeptides, as well as peptidomimetics, which can modulate the proliferation/differentiation state of epithelial cells by, as will be clear from the context of individual examples, mimicing or potentiating (agonizing) or inhibiting (antagonizing) the effects of a naturally-occurring *hedgehog* protein. A *hedgehog* therapeutic which mimics or potentiates the activity of a wild-type hedgehog protein is a "hedgehog agonist". Conversely, a *hedgehog* therapeutic which inhibits the activity of a wild-type hedgehog protein is a "hedgehog antagonist".

In particular, the term "hedgehog polypeptide" encompasses preparations of *hedgehog* proteins and peptidyl fragments thereof, both agonist and antagonist forms as the specific context will make clear.

As used herein the term "bioactive fragment of a *hedgehog* protein" refers to a fragment of a full-length *hedgehog* polypeptide, wherein the fragment specifically agonizes or antagonizes inductive events mediated by wild-type *hedgehog* proteins. The *hedgehog*

bioactive fragment preferably is a soluble extracellular portion of a *hedgehog* protein, where solubility is with reference to physiologically compatible solutions. Exemplary bioactive fragments are described in PCT publications WO 95/18856 and WO 96/17924.

The term "patched" or "ptc" refers to a family of related transmembrane proteins which have been implicated in the signal transduction induced by contacting a cell with a *hedgehog* protein. For example, the mammalian ptc family includes ptc1 and ptc2. In addition to references set out below, see also Takabatake et al. (1997) *FEBS Lett* 410:485 and GenBank AB000847 for examples of ptc2. Unless otherwise evident from the context, it will be understood that embodiments described in the context of ptc1 (or just ptc) also refer to equivalent embodiments involving other ptc homologs like ptc2.

The term "ptc therapeutic" refers to agents which either (i) mimic the effect of *hedgehog* proteins on *patched* signalling, e.g., which antagonize the cell-cycle inhibitory activity of *patched*, or (ii) activate or potentiate patched signalling. In other embodiments, the ptc therapeutic can be a *hedgehog* antagonist. The ptc therapeutic can be, e.g., a peptide. a nucleic acid, a carbohydrate, a small organic molecule, or natural product extract (or fraction thereof).

A "proliferative" form of a hedgehog or ptc therapeutic is one which induces proliferation of epithelial cells, particularly epithelial stem cells. Conversely, an "antiproliferative" form of a hedgehog or ptc therapeutic is one which inhibits proliferation of an epithelial cells, preferably in a non-toxic manner, e.g., by promoting or maintaining a differentiated phenotype or otherwise promoting quiescence.

By way of example, though not wishing to be bound by a particular theory, proliferative hedgehog polypeptide will generally be a form of the protein which derepresses patched-mediated cell-cycle arrest, e.g., the polypeptide mimics the effect of a naturally occurring hedgehog protein effect on epithelial cells. A proliferative ptc therapeutic includes other agents which depress patched-mediated cell-cycle arrest, and may act extracellularly or intracellularly.

An illustrative antiproliferative ptc therapeutic agent may potentiate patched-mediated cell-cycle arrest. Such agents can be small molecules that inhibit, e.g., hedgehog binding to

patched, as well as agents which stimulate and/or potentiate a signal transduction pathway of the patched protein.

The terms "epithelia", "epithelial" and "epithelium" refer to the cellular covering of internal and external body surfaces (cutaneous, mucous and serous), including the glands and other structures derived therefrom, e.g., corneal, esophegeal, epidermal, and hair follicle epithelial cells. Other exemplary epithlelial tissue includes: olfactory epithelium, which is the pseudostratified epithelium lining the olfactory region of the nasal cavity, and containing the receptors for the sense of smell; glandular epithelium, which refers to epithelium composed of secreting cells; squamous epithelium, which refers to epithelium composed of flattened plate-like cells. The term epithelium can also refer to transitional epithelium, which that characteristically found lining hollow organs that are subject to great mechanical change due to contraction and distention, e.g. tissue which represents a transition between stratified squamous and columnar epithelium.

The term "epithelialization" refers to healing by the growth of epithelial tissue over a denuded surface.

The term "skin" refers to the outer protective covering of the body, consisting of the corium and the epidermis, and is understood to include sweat and sebaceous glands, as well as hair follicle structures. Throughout the present application, the adjective "cutaneous" may be used, and should be understood to refer generally to attributes of the skin, as appropriate to the context in which they are used.

The term "epidermis" refers to the outermost and nonvascular layer of the skin, derived from the embryonic ectoderm, varying in thickness from 0.07-1.4 mm. On the palmar and plantar surfaces it comprises, from within outward, five layers: basal layer composed of columnar cells arranged perpendicularly; prickle-cell or spinous layer composed of flattened polyhedral cells with short processes or spines; granular layer composed of flattened granular cells; clear layer composed of several layers of clear, transparent cells in which the nuclei are indistinct or absent; and horny layer composed of flattened, cornified non-nucleated cells. In the epidermis of the general body surface, the clear layer is usually absent.

The "corium" or "dermis" refers to the layer of the skin deep to the epidermis, consisting of a dense bed of vascular connective tissue, and containing the nerves and terminal

organs of sensation. The hair roots, and sebaceous and sweat glands are structures of the epidermis which are deeply embedded in the dermis.

The term "nail" refers to the horny cutaneous plate on the dorsal surface of the distal end of a finger or toe.

The term "epidermal gland" refers to an aggregation of cells associated with the epidermis and specialized to secrete or excrete materials not related to their ordinary metabolic needs. For example, "sebaceous glands" are holocrine glands in the corium that secrete an oily substance and sebum. The term "sweat glands" refers to glands that secrete sweat, situated in the corium or subcutaneous tissue, opening by a duct on the body surface.

The term "hair" refers to a threadlike structure, especially the specialized epidermal structure composed of keratin and developing from a papilla sunk in the corium, produced only by mammals and characteristic of that group of animals. Also, the aggregate of such hairs. A "hair follicle" refers to one of the tubular-invaginations of the epidermis enclosing the hairs, and from which the hairs grow; and "hair follicle epithelial cells" refers to epithelial cells which surround the dermal papilla in the hair follicle, e.g., stem cells, outer root sheath cells, matrix cells, and inner root sheath cells. Such cells may be normal non-malignant cells, or transformed/immortalized cells.

The term "nasal epithelial tissue" refers to nasal and olfactory epithelium.

"Excisional wounds" include tears, abrasions, cuts, punctures or lacerations in the epithelial layer of the skin and may extend into the dermal layer and even into subcutaneous fat and beyond. Excisional wounds can result from surgical procedures or from accidental penetration of the skin.

"Burn wounds" refer to cases where large surface areas of skin have been removed or lost from an individual due to heat and/or chemical agents.

"Dermal skin ulcers" refer to lesions on the skin caused by superficial loss of tissue, usually with inflammation. Dermal skin ulcers which can be treated by the method of the present invention include decubitus ulcers, diabetic ulcers, venous stasis ulcers and arterial ulcers. Decubitus wounds refer to chronic ulcers that result from pressure applied to areas of the skin for extended periods of time. Wounds of this type are often called bedsores or

pressure sores. Venous stasis ulcers result from the stagnation of blood or other fluids from defective veins. Arterial ulcers refer to necrotic skin in the area around arteries having poor blood flow.

"Dental tissue" refers to tissue in the mouth which is similar to epithelial tissue, for example gum tissue. The method of the present invention is useful for treating periodontal disease.

"Internal epithelial tissue" refers to tissue inside the body which has characteristics similar to the epidermal layer in the skin. Examples include the lining of the intestine. The method of the present invention is useful for promoting the healing of certain internal wounds, for example wounds resulting from surgery.

A "wound to eye tissue" refers to severe dry eye syndrome, corneal ulcers and abrasions and ophthalmic surgical wounds.

Throughout this application, the term "proliferative skin disorder" refers to any disease/disorder of the skin marked by unwanted or aberrant proliferation of cutaneous tissue. These conditions are typically characterized by epidermal cell proliferation or incomplete cell differentiation, and include, for example, X-linked ichthyosis, psoriasis, atopic dermatitis, allergic contact dermatitis, epidermolytic hyperkeratosis, and seborrheic dermatitis. For example, epidermodysplasia is a form of faulty development of the epidermis.. Another example is "epidermolysis", which refers to a loosened state of the epidermis with formation of blebs and bullae either spontaneously or at the site of trauma.

The term "carcinoma" refers to a malignant new growth made up of epithelial cells tending to infiltrate surrounding tissues and to give rise to metastases. Exemplary carcinomas include: "basal cell carcinoma", which is an epithelial tumor of the skin that, while seldom metastasizing, has potentialities for local invasion and destruction; "squamous cell carcinoma", which refers to carcinomas arising from squamous epithelium and having cuboid cells; "carcinosarcoma", which include malignant tumors composed of carcinomatous and sarcomatous tissues; "adenocystic carcinoma", carcinoma marked by cylinders or bands of hyaline or mucinous stroma separated or surrounded by nests or cords of small epithelial cells, occurring in the mammary and salivary glands, and mucous glands of the respiratory tract; "epidermoid carcinoma", which refers to cancerous cells which tend to differentiate in the

same way as those of the epidermis; i.e., they tend to form prickle cells and undergo cornification; "nasopharyngeal carcinoma", which refers to a malignant tumor arising in the epithelial lining of the space behind the nose; and "renal cell carcinoma", which pertains to carcinoma of the renal parenchyma composed of tubular cells in varying arrangements. Another carcinomatous epithelial growth is "papillomas", which refers to benign tumors derived from epithelium and having a papillomavirus as a causative agent; and "epidermoidomas", which refers to a cerebral or meningeal tumor formed by inclusion of ectodermal elements at the time of closure of the neural groove.

As used herein, the term "psoriasis" refers to a hyperproliferative skin disorder which alters the skin's regulatory mechanisms. In particular, lesions are formed which involve primary and secondary alterations in epidermal proliferation, inflammatory responses of the skin, and an expression of regulatory molecules such as lymphokines and inflammatory factors. Psoriatic skin is morphologically characterized by an increased turnover of epidermal cells, thickened epidermis, abnormal keratinization, inflammatory cell infiltrates into the dermis layer and polymorphonuclear leukocyte infiltration into the epidermis layer resulting in an increase in the basal cell cycle. Additionally, hyperkeratotic and parakeratotic cells are present.

The term "keratosis" refers to proliferative skin disorder characterized by hyperplasia of the horny layer of the epidermis. Exemplary keratotic disorders include keratosis follicularis, keratosis palmaris et plantaris, keratosis pharyngea, keratosis pilaris, and actinic keratosis.

As used herein, "proliferating" and "proliferation" refer to cells undergoing mitosis.

As used herein, "transformed cells" refers to cells which have spontaneously converted to a state of unrestrained growth, i.e., they have acquired the ability to grow through an indefinite number of divisions in culture. Transformed cells may be characterized by such terms as neoplastic, anaplastic and/or hyperplastic, with respect to their loss of growth control.

As used herein, "immortalized cells" refers to cells which have been altered via chemical and/or recombinant means such that the cells have the ability to grow through an indefinite number of divisions in culture.

A "patient" or "subject" to be treated by the subject method can mean either a human or non-human animal.

The term "cosmetic preparation" refers to a form of a pharmaceutical preparation which is formulated for topical administration.

An "effective amount" of, e.g., a hedgehog therapeutic, with respect to the subject method of treatment, refers to an amount of, e.g., a hedgehog polypeptide in a preparation which, when applied as part of a desired dosage regimen brings about a change in the rate of cell proliferation and/or the state of differentiation of a cell so as to produce an amount of epithelial cell proliferation according to clinically acceptable standards for the disorder to be treated or the cosmetic purpose.

The "growth state" of a cell refers to the rate of proliferation of the cell and the state of differentiation of the cell.

"Homology" and "identity" each refer to sequence similarity between two polypeptide sequences, with identity being a more strict comparison. Homology and identity can each be determined by comparing a position in each sequence which may be aligned for purposes of comparison. When a position in the compared sequence is occupied by the same amino acid residue, then the polypeptides can be referred to as identical at that position; when the equivalent site is occupied by the same amino acid (e.g., identical) or a similar amino acid (e.g., similar in steric and/or electronic nature), then the molecules can be refered to as homologous at that position. A percentage of homology or identity between sequences is a function of the number of matching or homologous positions shared by the sequences. An "unrelated" or "non-homologous" sequence shares less than 40 percent identity, though preferably less than 25 percent identity, with an AR sequence of the present invention.

The term "corresponds to", when referring to a particular polypeptide or nucleic acid sequence is meant to indicate that the sequence of interest is identical or homologous to the reference sequence to which it is said to correspond.

The terms "recombinant protein", "heterologous protein" and "exogenous protein" are used interchangeably throughout the specification and refer to a polypeptide which is produced by recombinant DNA techniques, wherein generally, DNA encoding the polypeptide is

inserted into a suitable expression construct which is in turn used to transform a host cell to produce the heterologous protein. That is, the polypeptide is expressed from a heterologous nucleic acid.

A "chimeric protein" or "fusion protein" is a fusion of a first amino acid sequence encoding a *hedgehog* polypeptide with a second amino acid sequence defining a domain foreign to and not substantially homologous with any domain of *hh* protein. A chimeric protein may present a foreign domain which is found (albeit in a different protein) in an organism which also expresses the first protein, or it may be an "interspecies", "intergenic", etc. fusion of protein structures expressed by different kinds of organisms. In general, a fusion protein can be represented by the general formula $(X)_n$-$(hh)_m$-$(Y)_n$, wherein *hh* represents all or a portion of the *hedgehog* protein, X and Y each independently represent an amino acid sequences which are not naturally found as a polypeptide chain contiguous with the *hedgehog* sequence, m is an integer greater than or equal to 1, and each occurrence of n is, independently, 0 or an integer greater than or equal to 1 (n and m are preferably no greater than 5 or 10).

III. Exemplary Applications of Method and Compositions

The subject method has wide applicability to the treatment or prophylaxis of disorders afflicting epithelial tissue, as well as in cosmetic uses. In general, the method can be characterized as including a step of administering to an animal an amount of a *ptc* or hedgehog therapeutic effective to alter the proliferative state of a treated epithelial tissue. The mode of administration and dosage regimens will vary depending on the epithelial tissue(s) which is to be treated. For example, topical formulations will be preferred where the treated tissue is epidermal tissue, such as dermal or mucosal tissues. Likewise, as described in further detail below, the use of a particular ptc or hedgehog therapeutic, e.g., an agonist or antagonist, will depend on whether proliferation of cells of the treated tissue is desired or intended to be prevented.

A method which "promotes the healing of a wound" results in the wound healing more quickly as a result of the treatment than a similar wound heals in the absence of the treatment.

"Promotion of wound healing" can also mean that the method causes the proliferation and growth of, *inter alia*, keratinocytes, or that the wound heals with less scarring, less wound contraction, less collagen deposition and more superficial surface area. In certain instances, "promotion of wound healing" can also mean that certain methods of wound healing have improved success rates, (e.g. the take rates of skin grafts,) when used together with the method of the present invention.

Complications are a constant risk with wounds that have not fully healed and remain open. Although most wounds heal quickly without treatment, some types of wounds resist healing. Wounds which cover large surface areas also remain open for extended periods of time. In one embodiment of the present invention, the subject method can be used to accelerate the healing of wounds involving epithelial tissues, such as resulting from surgery, burns, inflammation or irritation. Certain of the hedgehog and ptc therapeutic formulations (e.g., proliferative forms) of the present invention can also be applied prophylactically, such as in the form of a cosmetic preparation, to enhance tissue regeneration processes, e.g., of the skin, hair and/or fingernails.

Despite significant progress in reconstructive surgical techniques, scarring can be an important obstacle in regaining normal function and appearance of healed skin. This is particularly true when pathologic scarring such as keloids or hypertrophic scars of the hands or face causes functional disability or physical deformity. In the severest circumstances, such scarring may precipitate psychosocial distress and a life of economic deprivation. Wound repair includes the stages of hemostasis, inflammation, proliferation, and remodeling. The proliferative stage involves multiplication of fibroblasts and endothelial and epithelial cells. Through the use of the subject method, the rate of proliferation of epithelial cells in and proximal to the wound can be controlled in order to accelerate closure of the wound and/or minimize the formation of scar tissue.

Full and partial thickness burns are an example of a wound type which often covers large surface areas and therefore requires prolonged periods of time to heal. As a result, life-threatening complications such as infection and loss of bodily fluids often arise. In addition, healing in burns is often disorderly, resulting in scarring and disfigurement. In some cases wound contraction due to excessive collagen deposition results in reduced mobility of muscles

in the vicinity of the wound. The compositions and method of the present invention can be used to accelerate the rate of healing of burns and to promote healing processes that result in more desirable cosmetic outcomes and less wound contraction and scarring.

Severe burns which cover large areas are often treated by skin autografts taken from undamaged areas of the patient's body. The subject method can also be used in conjunction with skin grafts to impove "take" rates of the graft by accelerating growth of both the grafted skin and the patient's skin that is proximal to the graft.

Dermal ulcers are yet another example of wounds that are amenable to treatment by the subject method, e.g., to cause healing of the ulcer and/or to prevent the ulcer from becoming a chronic wound. For example, one in seven individuals with diabetes develop dermal ulcers on their extremities, which are susceptible to infection. Individuals with infected diabetic ulcers often require hospitalization, intensive services, expensive antibiotics, and, in some cases, amputation. Dermal ulcers, such as those resulting from venous disease (venous stasis ulcers), excessive pressure (decubitus ulcers) and arterial ulcers also resist healing. The prior art treatments are generally limited to keeping the wound protected, free of infection and, in some cases, to restore blood flow by vascular surgery. According to the present method, the afflicted area of skin can be treated by a therapy which includes a *hedgehog* or ptc therapeutic which promotes epithelization of the wound, e.g., accelerates the rate of the healing of the skin ulcers.

The present treatment can also be effective as part of a therapeutic regimen for treating oral and paraoral ulcers, e.g. resulting from radiation and/or chemotherapy. Such ulcers commonly develop within days after chemotherapy or radiation therapy. These ulcers usually begin as small, painful irregularly shaped lesions usually covered by a delicate gray necrotic membrane and surrounded by inflammatory tissue. In many instances, lack of treatment results in proliferation of tissue around the periphery of the lesion on an inflammatory basis. For instance, the epithelium bordering the ulcer usually demonstrates proliferative activity, resulting in loss of continuity of surface epithelium. These lesions, because of their size and loss of epithelial integrity, lend the body to potential secondary infection. Routine ingestion of food and water becomes a very painful event and, if the ulcers proliferate throughout the alimentary canal, diarrhea usually is evident with all its complicating factors. According to the

present invention, a treatment for such ulcers which includes application of an hedgehog therapeutic can reduce the abnormal proliferation and differentiation of the affected epithelium, helping to reduce the severity of subsequent inflammatory events.

In another exemplary embodiment, the subject method is provided for treating or preventing gastrointestinal diseases. Briefly, a wide variety of diseases are associated with disruption of the gastrointestinal epithelium or villi, including chemotherapy- and radiation-therapy-induced enteritis (i.e. gut toxicity) and mucositis, peptic ulcer disease, gastroenteritis and colitis, villus atrophic disorders, and the like. For example, chemotherapeutic agents and radiation therapy used in bone marrow transplantation and cancer therapy affect rapidly proliferating cells in both the hematopoietic tissues and small intestine, leading to severe and often dose-limiting toxicities. Damage to the small intestine mucosal barrier results in serious complications of bleeding and sepsis. The subject method can be used to promote proliferation of gastrointenstinal epithelium and thereby increase the tolerated doses for radiation and chemotherapy agents. Effective treatment of gastrointestinal diseases may be determined by several criteria, including an enteritis score, other tests well known in the art.

The subject method and compositions can also be used to treat wounds resulting from dermatological diseases, such as lesions resulting from autoimmune disorders such as psoriasis. Atopic dermititis refers to skin trauma resulting from allergies associated with an immune response caused by allergens such as pollens, foods, dander, insect venoms and plant toxins.

With age, the epidermis thins and the skin appendages atrophy. Hair becomes sparse and sebaceous secretions decrease, with consequent susceptibility to dryness, chapping, and fissuring. The dermis diminishes with loss of elastic and collagen fibers. Moreover, keratinocyte proliferation (which is indicative of skin thickness and skin proliferative capacity) decreases with age. An increase in keratinocyte proliferation is believed to conteract skin aging, i.e., wrinkles, thickness, elasticity and repair. According to the present invention, a proliferative form of a *hedgehog* or ptc therapeutic can be used either therapeutically or cosmetically to counteract, at least for a time, the effects of aging on skin.

The subject method can also be used in treatment of a wound to eye tissue. Generally, damage to corneal tissue, whether by disease, surgery or injury, may affect epithelial and/or

endothelial cells, depending on the nature of the wound. Corneal epithelial cells are the non-keratinized epithelial cells lining the external surface of the cornea and provide a protective barrier against the external environment. Corneal wound healing has been of concern to both clinicians and researchers. Opthomologists are frequently confronted with corneal dystrophies and problematic injuries that result in persistent and recurrent epithelial erosion, often leading to permanent endothelial loss. The use of proliferative forms of the subject hedgehog and/or other ptc therapeutics can be used in these instances to promote epithelialization of the affected corneal tissue.

To further illustrate, specific disorders typically associated with epithelial cell damage in the eye, and for which the subject method can provide beneficial treatment, include persistent corneal epithelial defects, recurrent erosions, neurotrophic corneal ulcers, keratoconjunctivitis sicca, microbial corneal ulcers, viral cornea ulcers, and the like. Surgical procedures typically causing injury to the epithelial cell layers include laser procedures performed on the ocular surface, any refractive surgical procedures such as radial keratotomy and astigmatic keratotomy, conjunctival flaps, conjunctival transplants, epikeratoplasty, and corneal scraping. Moreover, superficial wounds such as scrapes, surface erosion, inflammation, etc. can cause lose of epithelial cells. According to the present invention, the corneal epithelium is contacted with an amount of a ptc or hedgehog therapeutic effective to cause proliferation of the corneal epithelial cells to appropriately heal the wound.

In other embodiments, antiproliferative preparations of hedgehog or ptc therapeutics can be used to inhibit lens epithelial cell proliferation to prevent post-operative complications of extracapsular cataract extraction. Cataract is an intractable eye disease and various studies on a treatment of cataract have been made. But at present, the treatment of cataract is attained by surgical operations. Cataract surgery has been applied for a long time and various operative methods have been examined. Extracapsular lens extraction has become the method of choice for removing cataracts. The major medical advantages of this technique over intracapsular extraction are lower incidence of aphakic cystoid macular edema and retinal detachment. Extracapsular extraction is also required for implantation of posterior chamber type intraocular lenses which are now considered to be the lenses of choice in most cases.

However, a disadvantage of extracapsular cataract extraction is the high incidence of posterior lens capsule opacification, often called after-cataract, which can occur in up to 50% of cases within three years after surgery. After-cataract is caused by proliferation of equatorial and anterior capsule lens epithelial cells which remain after extracapsular lens extraction. These cells proliferate to cause Sommerling rings, and along with fibroblasts which also deposit and occur on the posterior capsule, cause opacification of the posterior capsule, which interferes with vision. Prevention of after-cataract would be preferable to treatment. To inhibit secondary cataract formation, the subject method provides a means for inhibiting proliferation of the remaining lens epithelial cells. For example, such cells can be induced to remain quiescent by instilling a solution containing an antiproliferative hedgehog or ptc therapeutic preparation into the anterior chamber of the eye after lens removal. Furthermore, the solution can be osmotically balanced to provide minimal effective dosage when instilled into the anterior chamber of the eye, thereby inhibiting subcapsular epithelial growth with some specificity.

The subject method can also be used in the treatment of corneopathies marked by corneal epithelial cell proliferation, as for example in ocular epithelial disorders such as epithelial downgrowth or squamous cell carcinomas of the ocular surface.

The maintenance of tissues and organs *ex vivo* is also highly desirable. Tissue replacement therapy is well established in the treatment of human disease. For example, more than 40,000 corneal transplants were performed in the United States in 1996. Human epidermal cells can be grown in vitro and used to populate burn sites and chronic skin ulcers and other dermal wounds. The subject method can be used to accelerate the growth of epithelial tissue *in vitro*, as well as to accelerate the grafting of the cultured epithelial tissue to an animal host

The present method can be used for improving the "take rate" of a skin graft. Grafts of epidermal tissue can, if the take rate of the graft is to long, blister and shear, decreasing the likelihood that the autograft will "take", i.e. adhere to the wound and form a basement membrane with the underlying granulation tissue. Take rates can be increased by the subject method by inducing proliferation of the keratinocytes. The method of increasing take rates comprises contacting the skin autograft with an effective wound healing amount of a hedgehog

or ptc therapeutic compositions described in the method of promoting wound healing and in the method of promoting the growth and proliferation of keratinocytes, as described above.

Skin equivalents have many uses not only as a replacement for human or animal skin for skin grafting, but also as test skin for determining the effects of pharmaceutical substances and cosmetics on skin. A major difficulty in pharmacological, chemical and cosmetic testing is the difficulties in determining the efficacy and safety of the products on skin. One advantage of the skin equivalents of the invention is their use as an indicator of the effects produced by such substances through in vitro testing on test skin.

Thus, in one embodiment of the subject method can be used as part of a protocol for skin grafting of, e.g., denuded areas, granulating wounds and burns. The use of proliferative hedgehog and/or ptc therapeutics can enhance such grafting techniques as split thickness autografts and epidermal autografts (cultured autogenic keratinocytes) and epidermal allografts (cultured allogenic keratinocytes). In the instance of the allograft, the use of the subject method to enhance the formation of skin equivalents in culture helps to provide/maintain a ready supply of such grafts (e.g., in tissue banks) so that the patients might be covered in a single procedure with a material which allows permanent healing to occur.

In this regard, the present invention also concerns composite living skin equivalents comprising an epidermal layer of cultured keratinocyte cells which have been expanded by treatment with a hedgehog or other ptc therapeutic. The subject method can be used as part of a process for the preparation of composite living skin equivalents. In an illustrative embodiment, such a method comprises obtaining a skin sample, treating the skin sample enzymically to separate the epidermis from the dermis, treating the epidermis enzymically to release the keratinocyte cells, culturing, in the presence of a hedgehog or ptc therapeutic, the epidermal keratinocytes until confluence, in parallel, or separately, treating the dermis enzymatically to release the fibroblast cells, culturing the fibroblasts cells until sub-confluence, inoculating a porous, cross-linked collagen sponge membrane with the cultured fibroblast cells, incubating the inoculated collagen sponge on its surface to allow the growth of the fibroblast cells throughout the collagen sponge, and then inoculating it with cultured keratinocyte cells, and further incubating the composite skin equivalent complex in the presence of a hedgehog or ptc therapeutic to promote the growth of the cells.

In other embodiments, skin sheets containing both epithelial and mesenchymal layers can be isolated in culture and expanded with culture media supplemented with a proliferative form of a hedgehog or ptc therapeutic.

Any skin sample amenable to cell culture techniques can be used in accordance with the present invention. The skin samples may be autogenic or allogenic.

In another aspect of the invention, the subject method can be used in conjunction with various periodontal procedures in which control of epithelial cell proliferation in and around periodontal tissue is desired.

In one embodiment, proliferative forms of the hedgehog and ptc therapeutics can be used to enhance reepithelialization around natural and prosthetic teeth, e.g., to promote formation of gum tissue.

In another embodiment, antiproliferative ptc therapeutics can find application in the treatment of peridontal disease. It is estimated that in the United States alone, there are in excess of 125 million adults with periodontal disease in varying forms. Periodontal disease starts as inflammatory lesions because of specific bacteria localizing in the area where the gingiva attaches to the tooth. Usually first to occur is a vascular change in the underlying connective tissue. Inflammation in the connective tissue stimulates the following changes in the epithelial lining of the sulcus and in the epithelial attachment: increased mitotic activity in the basal epithelial layer; increased producing of keratin with desquamation; cellular desquamation adjacent to the tooth surface tends to deepen the pocket; epithelial cells of the basal layer at the bottom of the sulcus and in the area of attachment proliferate into the connective tissue and break up of the gingival fibers begins to occur, wherein dissolution of the connective tissue results in the formation of an open lesion. The application of hedgehog preparations to the periodontium can be used to inhibit proliferation of epithelial tissue and thus prevent further periodontoclastic development.

In yet another aspect, the subject method can be used to help control guided tissue regeneration, such as when used in conjunction with bioresorptable materials. For example, incorporation of periodontal implants, such as prosthetic teeth, can be facilitated by the instant method. Reattachment of a tooth involves both formation of connective tissue fibers and re-epithelization of the tooth pocket. The subject methodtreatment can be used to accelerate

tissue reattachment by controlling the mitotic function of basal epithelial cells in early stages of wound healing.

Yet another aspect of the present invention relates to the use of hedgehog therapeutic preparations to control hair growth. Hair is basically composed of keratin, a tough and insoluble protein; its chief strength lies in its disulphide bond of cystine. Each individual hair comprises a cylindrical shaft and a root, and is contained in a follicle, a flask-like depression in the skin. The bottom of the follicle contains a finger-like projection termed the papilla, which consists of connective tissue from which hair grows, and through which blood vessels supply the cells with nourishment. The shaft is the part that extends outwards from the skin surface, whilst the root has been described as the buried part of the hair. The base of the root expands into the hair bulb, which rests upon the papilla. Cells from which the hair is produced grow in the bulb of the follicle; they are extruded in the form of fibers as the cells proliferate in the follicle. Hair "growth" refers to the formation and elongation of the hair fiber by the dividing cells.

As is well known in the art, the common hair cycle is divided into three stages: anagen, catagen and telogen. During the active phase (anagen) , the epidermal stem cells of the dermal papilla divide rapidly. Daughter cells move upward and differentiate to form the concentric layers of the hair itself. The transitional stage, catagen, is marked by the cessation of mitosis of the stem cells in the follicle. The resting stage is known as telogen, where the hair is retained within the scalp for several weeks before an emerging new hair developing below it dislodges the telogen-phase shaft from its follicle. From this model it has become clear that the larger the pool of dividing stem cells that differentiate into hair cells, the more hair growth occurs. Accordingly, methods for increasing or reducing hair growth can be carried out by potentiating or inhibiting, respectively, the proliferation of these stem cells.

In one embodiment, the subject method provides a means for altering the dynamics of the hair growth cycle to induce proliferation of hair follicle cells, particularly stem cells of the hair follicle. The subject compositions and method can be used to increase hair follicle size and the rate of hair growth in warm-blooded animals, such as humans, e.g., by promoting proliferation of hair follicle stem cells. In one embodiment, the method comprises administering to the skin in the area in which hair growth is desired an amount of hedgehog or

ptc therapeutic sufficient to increase hair follicle size and/or the rate of hair growth in the animal. Typically, the composition will be administered topically as a cream, and will be applied on a daily basis until hair growth is observed and for a time thereafter sufficient to maintain the desired amount of hair growth. This method can have applications in the promotion of new hair growth or stimulation of the rate of hair growth, e.g., following chemotherapeutic treatment or for treating various forms of alopecia, e.g., male pattern baldness. For instance, one of several biochemical cellular and molecular disturbances that occur during the anagen phase or catagen phase of subjects with androgenic alopecia can be corrected or improved by treatment using the subject method, e.g., in the functioning or formation of the stem cells, their migration process or during the mitosis phase of keratin production within the follicular papilla and matrix.

In other embodimemts, cerain of the hedgehog and ptc therapeutics (e.g., antiproliferative forms) can be employed as a way of reducing the growth of human hair as opposed to its conventional removal by cutting, shaving, or depilation. For instance, the present method can be used in the treatment of trichosis characterized by abnormally rapid or dense growth of hair, e.g. hypertrichosis. In an exemplary embodiment, hedgehog antagonists can be used to manage hirsutism, a disorder marked by abnormal hairiness. The subject method can also provide a process for extending the duration of depilation.

Moreover, because a hedgehog antagonist (or ptc agonist) will often be cytostatic to epithelial cells, rather than cytotoxic, such agents can be used to protect hair follicle cells from cytotoxic agents which require progression into S-phase of the cell-cycle for efficacy, e.g. radiation-induced death. Treatment by the subject method can provide protection by causing the hair follicle cells to become quiescent, e.g., by inhibiting the cells from entering S phase, and thereby preventing the follicle cells from undergoing mitotic catastrophe or programmed cell death. For instance, hedgehog antagonists can be used for patients undergoing chemo- or radiation-therapies which ordinarily result in hair loss. By inhibiting cell-cycle progression during such therapies, the subject treatment can protect hair follicle cells from death which might otherwise result from activation of cell death programs. After the therapy has concluded, the hedgehog or ptc treatment can also be removed with concommitant relief of the inhibition of follicle cell proliferation.

The subject method can also be used in the treatment of folliculitis, such as folliculitis decalvans, folliculitis ulerythematosa reticulata or keloid folliculitis. For example, a cosmetic prepration of an hedgehog therapeutic can be applied topically in the treatment of pseudofolliculitis, a chronic disorder occurring most often in the submandibular region of the neck and associated with shaving, the characteristic lesions of which are erythematous papules and pustules containing buried hairs.

In another aspect of the invention, antiproliferative forms of the subject *hedgehog* and ptc therapeutics can be used to induce differentiation of epithelially-derived tissue. Such forms of these molecules can provide a basis for differentiation therapy for the treatment of hyperplastic and/or neoplastic conditions involving epithelial tissue. For example, such preparations can be used for the treatment of cutaneous diseases in which there is abnormal proliferation or growth of cells of the skin.

For instance, the pharmaceutical preparations of the invention are intended for the treatment of hyperplastic epidermal conditions, such as keratosis, as well as for the treatment of neoplastic epidermal conditions such as those characterized by a high proliferation rate for various skin cancers, as for example basal cell carcinoma or squamous cell carcinoma. The subject method can also be used in the treatment of autoimmune diseases affecting the skin, in particular, of dermatological diseases involving morbid proliferation and/or keratinization of the epidermis, as for example, caused by psoriasis or atopic dermatosis.

Many common diseases of the skin, such as psoriasis, squamous cell carcinoma, keratoacanthoma and actinic keratosis are characterized by localized abnormal proliferation and growth. For example, in psoriasis, which is characterized by scaly, red, elevated plaques on the skin, the keratinocytes are known to proliferate much more rapidly than normal and to differentiate less completely.

In one embodiment, the preparations of the present invention are suitable for the treatment of dermatological ailments linked to keratinization disorders causing abnormal proliferation of skin cells, which disorders may be marked by either inflammatory or non-inflammatory components. To illustrate, therapeutic preparations of a ptc agonist, e.g., which promotes quiescense or differentiation can be used to treat varying forms of psoriasis, be they cutaneous, mucosal or ungual. Psoriasis, as described above, is typically characterized by

epidermal keratinocytes which display marked proliferative activation and differentiation along a "regenerative" pathway. Treatment with an antiproliferative embodiment of the subject method can be used to reverse the pathological epidermal activiation and can provide a basis for sustained remission of the disease.

A variety of other keratotic lesions are also candidates for treatment with the subject antiproliferative preparations. Actinic keratoses, for example, are superficial inflammatory premalignant tumors arising on sun-exposed and irradiated skin. The lesions are erythematous to brown with variable scaling. Current therapies include excisional and cryosurgery. These treatments are painful, however, and often produce cosmetically unacceptable scarring. Accordingly, treatment of keratosis, such as actinic keratosis, can include application, preferably topical, of a ptc agonist composition in amounts sufficient to inhibit hyperproliferation of epidermal/epidermoid cells of the lesion.

Acne represents yet another dermatologic ailment which may be treated with an antiproliferative embodiment of the subject method. Acne vulgaris, for instance, is a multifactorial disease most commonly occurring in teenagers and young adults, and is characterized by the appearance of inflammatory and noninflammatory lesions on the face and upper trunk. The basic defect which gives rise to acne vulgaris is hypercornification of the duct of a hyperactive sebaceous gland. Hypercornification blocks the normal mobility of skin and follicle microorganisms, and in so doing, stimulates the release of lipases by Propinobacterium acnes and *Staphylococcus epidermidis* bacteria and *Pitrosporum ovale*, a yeast. Treatment with an antiproliferative form of a hedgehog or ptc therapeutic, particularly topical preparations, may be useful for preventing the transitional features of the ducts, e.g. hypercornification, which lead to lesion formation. The subject treatment may further include, for example, antibiotics, retinoids and antiandrogens.

The present invention also provides a method for treating various forms of dermatitis. Dermatitis is a descriptive term referring to poorly demarcated lesions which are either pruritic, erythematous, scaley, blistered, weeping, fissured or crusted. These lesions arise from any of a wide variety of causes. The most common types of dermatitis are atopic, contact and diaper dermatitis. For instance, seborrheic dermatitis is a chronic, usually pruritic, dermatitis with erythema, dry, moist, or greasy scaling, and yellow crusted patches on various areas,

especially the scalp, with exfoliation of an excessive amount of dry scales stasis dermatitis, an often chronic, usually eczematous dermatitis. Actinic dermatitis is dermatitis that due to exposure to actinic radiation such as that from the sun, ultraviolet waves or x- or gamma-radiation. According to the present invention, the subject hedgehog or ptc therapeutic preparations can be used in the treatment and/or prevention of certain symptoms of dermatitis caused by unwanted proliferation of epithelial cells. Such therapies for these various forms of dermatitis can also include topical and systemic corticosteroids, antipuritics, and antibiotics.

Also included in ailments which may be treated by the subject method are disorders specific to non-humans, such as mange.

IV. Exemplary hedgehog therapeutic compounds.

The *hedgehog* therapeutic compositions of the subject method can be generated by any of a variety of techniques, including purification of naturally occurring proteins, recombinantly produced proteins and synthetic chemistry. Polypeptide forms of the hedgehog therapeutics are preferably derived from vertebrate hedgehog proteins, e.g., have sequences corresponding to naturally occurring hedgehog proteins, or fragments thereof, from vertebrate organisms. However, it will be appreciated that the hedgehog polypeptide can correspond to a hedgehog protein (or fragment thereof) which occurs in any metazoan organism.

The various naturally-occurring *hedgehog* proteins from which the subject therapeutics can be derived are characterized by a signal peptide, a highly conserved N-terminal region, and a more divergent C-terminal domain. In addition to signal sequence cleavage in the secretory pathway (Lee, J.J. *et al.* (1992) *Cell* 71:33-50; Tabata, T. *et al.* (1992) *Genes Dev.* 2635-2645; Chang, D.E. *et al.* (1994) *Development* 120:3339-3353), *hedgehog* precursor proteins naturally undergo an internal autoproteolytic cleavage which depends on conserved sequences in the C-terminal portion (Lee *et al.* (1994) *Science* 266:1528-1537; Porter *et al.* (1995) *Nature* 374:363-366). This autocleavage leads to a 19 kD N-terminal peptide and a C-terminal peptide of 26-28 kD (Lee *et al.* (1992) *supra*; Tabata *et al.* (1992) *supra*; Chang *et al.* (1994) *supra*; Lee *et al.* (1994) *supra*; Bumcrot, D.A., *et al.* (1995) *Mol. Cell. Biol.* 15:2294-2303; Porter *et al.* (1995) *supra*; Ekker, S.C. *et al.* (1995) *Curr. Biol.* 5:944-955; Lai, C.J. *et al.* (1995) *Development* 121:2349-2360). The N-terminal peptide stays tightly associated with the

surface of cells in which it was synthesized, while the C-terminal peptide is freely diffusible both *in vitro* and *in vivo* (Lee *et al.* (1994) *supra*; Bumcrot *et al.* (1995) *supra;* Mart', E. *et al.* (1995) *Development* 121:2537-2547; Roelink, H. *et al.* (1995) *Cell* 81:445-455). Cell surface retention of the N-terminal peptide is dependent on autocleavage, as a truncated form of *hedgehog* encoded by an RNA which terminates precisely at the normal position of internal cleavage is diffusible *in vitro* (Porter *et al.* (1995) *supra*) and *in vivo* (Porter, J.A. *et al.* (1996) *Cell* 86, 21-34). Biochemical studies have shown that the autoproteolytic cleavage of the *hedgehog* precursor protein proceeds through an internal thioester intermediate which subsequently is cleaved in a nucleophilic substitution. It is suggested that the nucleophile is a small lipophilic molecule, more particularly cholesterol, which becomes covalently bound to the C-terminal end of the N-peptide (Porter *et al.* (1996) *supra*), tethering it to the cell surface.

The vertebrate family of *hedgehog* genes includes at least four members, e.g., paralogs of the single drosophila *hedgehog* gene (SEQ ID No. 19). Three of these members, herein referred to as Desert *hedgehog* (*Dhh*), Sonic *hedgehog* (*Shh*) and Indian *hedgehog* (*Ihh*), apparently exist in all vertebrates, including fish, birds, and mammals. A fourth member, herein referred to as tiggie-winkle *hedgehog* (*Thh*), appears specific to fish.. According to the appended sequence listing, (see also Table 1) a chicken *Shh* polypeptide is encoded by SEQ ID NO:1; a mouse *Dhh* polypeptide is encoded by SEQ ID NO:2; a mouse *Ihh* polypeptide is encoded by SEQ ID NO:3; a mouse *Shh* polypeptide is encoded by SEQ ID NO:4 a zebrafish *Shh* polypeptide is encoded by SEQ ID NO:5; a human *Shh* polypeptide is encoded by SEQ ID NO:6; a human *Ihh* polypeptide is encoded by SEQ ID NO:7; a human *Dhh* polypeptide is encoded by SEQ ID No. 8; and a zebrafish *Thh* is encoded by SEQ ID No. 9.

Table 1
Guide to *hedgehog* sequences in Sequence Listing

	Nucleotide	Amino Acid
Chicken *Shh*	SEQ ID No. 1	SEQ ID No. 10
Mouse *Dhh*	SEQ ID No. 2	SEQ ID No. 11
Mouse *Ihh*	SEQ ID No. 3	SEQ ID No. 12
Mouse *Shh*	SEQ ID No. 4	SEQ ID No. 13
Zebrafish *Shh*	SEQ ID No. 5	SEQ ID No. 14
Human *Shh*	SEQ ID No. 6	SEQ ID No. 15
Human *Ihh*	SEQ ID No. 7	SEQ ID No. 16
Human *Dhh*	SEQ ID No. 8	SEQ ID No. 17
Zebrafish *Thh*	SEQ ID No. 9	SEQ ID No. 18
Drosophila *HH*	SEQ ID No. 19	SEQ ID No. 20

In addition to the sequence variation between the various *hedgehog* homologs, the *hedgehog* proteins are apparently present naturally in a number of different forms, including a pro-form, a full-length mature form, and several processed fragments thereof. The pro-form includes an N-terminal signal peptide for directed secretion of the extracellular domain, while the full-length mature form lacks this signal sequence.

As described above, further processing of the mature form occurs in some instances to yield biologically active fragments of the protein. For instance, *sonic hedgehog* undergoes additional proteolytic processing to yield two peptides of approximately 19 kDa and 27 kDa, the 19kDa fragment corresponding to an proteolytic N-terminal portion of the mature protein.

In addition to proteolytic fragmentation, the vertebrate *hedgehog* proteins can also be modified post-translationally, such as by glycosylation and/or addition of lipophilic moieties, such as stents, fatty acids, etc., though bacterially produced (e.g. unmodified) forms of the proteins still maintain certain of the bioactivities of the native protein. Bioactive fragments of *hedgehog* polypeptides of the present invention have been generated and are described in great detail in, e.g., PCT publications WO 95/18856 and WO 96/17924.

There are a wide range of lipophilic moieties with which hedgehog polypeptides can be derivatived. The term "lipophilic group", in the context of being attached to a hedgehog polypeptide, refers to a group having high hydrocarbon content thereby giving the group high affinity to lipid phases. A lipophilic group can be, for example, a relatively long chain alkyl or cycloalkyl (preferably n-alkyl) group having approximately 7 to 30 carbons. The alkyl group

may terminate with a hydroxy or primary amine "tail". To further illustrate, lipophilic molecules include naturally-occurring and synthetic aromatic and non-aromatic moieties such as fatty acids, sterols, esters and alcohols, other lipid molecules, cage structures such as adamantane and buckminsterfullerenes, and aromatic hydrocarbons such as benzene, perylene, phenanthrene, anthracene, naphthalene, pyrene, chrysene, and naphthacene.

In one embodiment, the hedgehog polypeptide is modified with one or more sterol moieties, such as cholesterol. See, for example, PCT publication WO 96/17924. In certain embodiments, the cholesterol is preferably added to the C-terminal glycine were the hedgehog polypeptide corresponds to the naturally-occurring N-terminal proteolytic fragment.

In another embodiment, the hedgehog polypeptide can be modified with a fatty acid moiety, such as a myrostoyl, palmitoyl, stearoyl, or arachidoyl moiety. See, e.g., Pepinsky et al. (1998) J Biol. Chem 273: 14037.

In addition to those effects seen by cholesterol-addition to the C-terminus or fatty acid addition to the N-terminus of extracellular fragments of the protein, at least certain of the biological activities of the hedgehog gene products are unexpectedly potentiated by derivatization of the protein with lipophilic moieties at other sites on the protein and/or by moieties other than cholesterol or fatty acids. Certain aspects of the invention are directed to the use of preparations of hedgehog polypeptides which are modified at sites other than N-terminal or C-terminal residues of the natural processed form of the protein, and/or which are modified at such terminal residues with lipophilic moieties other than a sterol at the C-terminus or fatty acid at the N-terminus.

Particularly useful as lipophilic molecules are alicyclic hydrocarbons, saturated and unsaturated fatty acids and other lipid and phospholipid moieties, waxes, cholesterol, isoprenoids, terpenes and polyalicyclic hydrocarbons including adamantane and buckminsterfullerenes, vitamins, polyethylene glycol or oligoethylene glycol, (C1-C18)-alkyl phosphate diesters, -O-CH2-CH(OH)-O-(C12-C18)-alkyl, and in particular conjugates with pyrene derivatives. The lipophilic moiety can be a lipophilic dye suitable for use in the invention include, but are not limited to, diphenylhexatriene, Nile Red, N-phenyl-1-naphthylamine, Prodan, Laurodan, Pyrene, Perylene, rhodamine, rhodamine B, tetramethylrhodamine, Texas Red, sulforhodamine, 1,1'-didodecyl-

3,3,3',3'tetramethylindocarbocyanine perchlorate, octadecyl rhodamine B and the BODIPY dyes available from Molecular Probes Inc.

Other exemplary lipophilic moietites include aliphatic carbonyl radical groups include 1- or 2-adamantylacetyl, 3-methyladamant-1-ylacetyl, 3-methyl-3-bromo-1-adamantylacetyl, 1-decalinacetyl, camphoracetyl, camphaneacetyl, noradamantylacetyl, norbornaneacetyl, bicyclo[2.2.2.]-oct-5-eneacetyl, 1-methoxybicyclo[2.2.2.]-oct-5-ene-2-carbonyl, cis-5-norbornene-endo-2,3-dicarbonyl, 5-norbornen-2-ylacetyl, (1R)-(-)-myrtentaneacetyl, 2-norbornaneacetyl, anti-3-oxo-tricyclo[2.2:1.0<2,6> .]-heptane-7-carbonyl, decanoyl, dodecanoyl, dodecenoyl, tetradecadienoyl, decynoyl or dodecynoyl.

The hedgehog polypeptide can be linked to the hydrophobic moiety in a number of ways including by chemical coupling means, or by genetic engineering.

There are a large number of chemical cross-linking agents that are known to those skilled in the art. For the present invention, the preferred cross-linking agents are heterobifunctional cross-linkers, which can be used to link the hedgehog polypeptide and hydrophobic moiety in a stepwise manner. Heterobifunctional cross-linkers provide the ability to design more specific coupling methods for conjugating to proteins, thereby reducing the occurrences of unwanted side reactions such as homo-protein polymers. A wide variety of heterobifunctional cross-linkers are known in the art. These include: succinimidyl 4-(N-maleimidomethyl) cyclohexane- 1-carboxylate (SMCC), m-Maleimidobenzoyl-N-hydroxysuccinimide ester (MBS); N-succinimidyl (4-iodoacetyl) aminobenzoate (SIAB), succinimidyl 4-(p-maleimidophenyl) butyrate (SMPB), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC); 4-succinimidyloxycarbonyl- a-methyl-a-(2-pyridyldithio)-tolune (SMPT), N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP), succinimidyl 6-[3-(2-pyridyldithio) propionate] hexanoate (LC-SPDP). Those cross-linking agents having N-hydroxysuccinimide moieties can be obtained as the N-hydroxysulfosuccinimide analogs, which generally have greater water solubility. In addition, those cross-linking agents having disulfide bridges within the linking chain can be synthesized instead as the alkyl derivatives so as to reduce the amount of linker cleavage *in vivo*.

In addition to the heterobifunctional cross-linkers, there exists a number of other cross-linking agents including homobifunctional and photoreactive cross-linkers. Disuccinimidyl

suberate (DSS), bismaleimidohexane (BMH) and dimethylpimelimidate·2 HCl (DMP) are examples of useful homobifunctional cross-linking agents, and bis-[ß-(4-azidosalicylamido)ethyl]disulfide (BASED) and N-succinimidyl-6(4'-azido-2'-nitrophenyl-amino)hexanoate (SANPAH) are examples of useful photoreactive cross-linkers for use in this invention. For a recent review of protein coupling techniques, see Means et al. (1990) *Bioconjugate Chemistry* 1:2-12, incorporated by reference herein.

One particularly useful class of heterobifunctional cross-linkers, included above, contain the primary amine reactive group, N-hydroxysuccinimide (NHS), or its water soluble analog N-hydroxysulfosuccinimide (sulfo-NHS). Primary amines (lysine epsilon groups) at alkaline pH's are unprotonated and react by nucleophilic attack on NHS or sulfo-NHS esters. This reaction results in the formation of an amide bond, and release of NHS or sulfo-NHS as a by-product.

Another reactive group useful as part of a heterobifunctional cross-linker is a thiol reactive group. Common thiol reactive groups include maleimides, halogens, and pyridyl disulfides. Maleimides react specifically with free sulfhydryls (cysteine residues) in minutes, under slightly acidic to neutral (pH 6.5-7.5) conditions. Halogens (iodoacetyl functions) react with -SH groups at physiological pH's. Both of these reactive groups result in the formation of stable thioether bonds.

The third component of the heterobifunctional cross-linker is the spacer arm or bridge. The bridge is the structure that connects the two reactive ends. The most apparent attribute of the bridge is its effect on steric hindrance. In some instances, a longer bridge can more easily span the distance necessary to link two complex biomolecules. For instance, SMPB has a span of 14.5 angstroms.

Preparing protein-protein conjugates using heterobifunctional reagents is a two-step process involving the amine reaction and the sulfhydryl reaction. For the first step, the amine reaction, the protein chosen should contain a primary amine. This can be lysine epsilon amines or a primary alpha amine found at the N-terminus of most proteins. The protein should not contain free sulfhydryl groups. In cases where both proteins to be conjugated contain free sulfhydryl groups, one protein can be modified so that all sulfhydryls are blocked using for instance, N-ethylmaleimide (see Partis et al. (1983) J. Pro. Chem. 2:263,

incorporated by reference herein). Ellman's Reagent can be used to calculate the quantity of sulfhydryls in a particular protein (see for example Ellman et al. (1958) Arch. Biochem. Biophys. 74:443 and Riddles et al. (1979) Anal. Biochem. 94:75, incorporated by reference herein).

The reaction buffer should be free of extraneous amines and sulfhydryls. The pH of the reaction buffer should be 7.0-7.5. This pH range prevents maleimide groups from reacting with amines, preserving the maleimide group for the second reaction with sulfhydryls.

The NHS-ester containing cross-linkers have limited water solubility. They should be dissolved in a minimal amount of organic solvent (DMF or DMSO) before introducing the cross-linker into the reaction mixture. The cross-linker/solvent forms an emulsion which will allow the reaction to occur.

The sulfo-NHS ester analogs are more water soluble, and can be added directly to the reaction buffer. Buffers of high ionic strength should be avoided, as they have a tendency to "salt out" the sulfo-NHS esters. To avoid loss of reactivity due to hydrolysis, the cross-linker is added to the reaction mixture immediately after dissolving the protein solution.

The reactions can be more efficient in concentrated protein solutions. The more alkaline the pH of the reaction mixture, the faster the rate of reaction. The rate of hydrolysis of the NHS and sulfo-NHS esters will also increase with increasing pH. Higher temperatures will increase the reaction rates for both hydrolysis and acylation.

Once the reaction is completed, the first protein is now activated, with a sulfhydryl reactive moiety. The activated protein may be isolated from the reaction mixture by simple gel filtration or dialysis. To carry out the second step of the cross-linking, the sulfhydryl reaction, the lipophilic group chosen for reaction with maleimides, activated halogens, or pyridyl disulfides must contain a free sulfhydryl. Alternatively, a primary amine may be modified with to add a sulfhydryl

In all cases, the buffer should be degassed to prevent oxidation of sulfhydryl groups. EDTA may be added to chelate any oxidizing metals that may be present in the buffer. Buffers should be free of any sulfhydryl containing compounds.

Maleimides react specifically with -SH groups at slightly acidic to neutral pH ranges (6.5-7.5). A neutral pH is sufficient for reactions involving halogens and pyridyl disulfides. Under these conditions, maleimides generally react with -SH groups within a matter of minutes. Longer reaction times are required for halogens and pyridyl disulfides.

The first sulfhydryl reactive-protein prepared in the amine reaction step is mixed with the sulfhydryl-containing lipophilic group under the appropriate buffer conditions. The conjugates can be isolated from the reaction mixture by methods such as gel filtration or by dialysis.

Exemplary activated lipophilic moieties for conjugation include: N-(1-pyrene)maleimide; 2,5-dimethoxystilbene-4'-maleimide, eosin-5-maleimide; fluorescein-5-maleimide; N-(4-(6-dimethylamino- 2-benzofuranyl)phenyl)maleimide; benzophenone-4-maleimide; 4-dimethylaminophenylazophenyl- 4'-maleimide (DABMI), tetramethylrhodamine-5-maleimide, tetramethylrhodamine-6-maleimide, Rhodamine RedTM C2 maleimide, N-(5-aminopentyl)maleimide, trifluoroacetic acid salt, N-(2-aminoethyl)maleimide, trifluoroacetic acid salt, Oregon GreenTM 488 maleimide, N-(2-((2-(((4-azido- 2,3,5,6-tetrafluoro)benzoyl) amino)ethyl)dithio)ethyl)maleimide (TFPAM-SS1), 2-(1-(3-dimethylaminopropyl) -indol-3-yl)-3-(indol-3-yl) maleimide (bisindolylmaleimide; GF 109203X), BODIPY® FL N-(2-aminoethyl)maleimide, N-(7-dimethylamino- 4-methylcoumarin-3-yl)maleimide (DACM), AlexaTM 488 C5 maleimide, AlexaTM 594 C5 maleimide, sodium saltN-(1-pyrene)maleimide, 2,5-dimethoxystilbene-4'-maleimide, eosin-5-maleimide, fluorescein-5-maleimide, N-(4-(6-dimethylamino- 2-benzofuranyl)phenyl)maleimide, benzophenone-4-maleimide, 4-dimethylaminophenylazophenyl- 4'-maleimide, 1-(2-maleimidylethyl)-4-(5- (4-methoxyphenyl)oxazol-2- yl)pyridinium methanesulfonate, tetramethylrhodamine-5-maleimide, tetramethylrhodamine-6-maleimide, Rhodamine RedTM C2 maleimide, N-(5-aminopentyl)maleimide, N-(2-aminoethyl)maleimide, N-(2-((2-(((4-azido- 2,3,5,6-tetrafluoro)benzoyl) amino)ethyl)dithio)ethyl)maleimide, 2-(1-(3-dimethylaminopropyl) -indol-3-yl)-3-(indol-3-yl) maleimide, N-(7-dimethylamino- 4-methylcoumarin-3-yl)maleimide (DACM), 11H-Benzo[a]fluorene, Benzo[a]pyrene.

In one embodiment, the hedgehog polypeptide can be derivatived using pyrene maleimide, which can be purchased from Molecular Probes (Eugene, Oreg.), e.g., N-(1-pyrene)maleimide or 1-pyrenemethyl iodoacetate (PMIA ester).

For those embodiments wherein the hydophobic moiety is a polypeptide, the modified hedgehog polypeptide of this invention can be constructed as a fusion protein, containing the hedgehog polypeptide and the hydrophobic moiety as one contiguous polypeptide chain.

In certain embodiments, the lipophilic moiety is an amphipathic polypeptide, such as magainin, cecropin, attacin, melittin, gramicidin S, alpha-toxin of Staph. aureus, alamethicin or a synthetic amphipathic polypeptide. Fusogenic coat proteins from viral particles can also be a convenient source of amphipathic sequences for the subject hedgehog proteins

Moreover, mutagenesis can be used to create modified *hh* polypeptides, e.g., for such purposes as enhancing therapeutic or prophylactic efficacy, or stability (e.g., *ex vivo* shelf life and resistance to proteolytic degradation *in vivo*). Such modified peptides can be produced, for instance, by amino acid substitution, deletion, or addition. Modified *hedgehog* polypeptides can also include those with altered post-translational processing relative to a naturally occurring *hedgehog* protein, e.g., altered glycosylation, cholesterolization, prenylation and the like.

In one embodiment, the hedgehog therapeutic is a polypeptide encodable by a nucleotide sequence that hybridizes under stringent conditions to a hedgehog coding sequence represented in one or more of SEQ ID NOs:1-7. Appropriate stringency conditions which promote DNA hybridization, for example, 6.0 x sodium chloride/sodium citrate (SSC) at about 45°C, followed by a wash of 2.0 x SSC at 50°C, are known to those skilled in the art or can be found in *Current Protocols in Molecular Biology*, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. For example, the salt concentration in the wash step can be selected from a low stringency of about 2.0 x SSC at 50°C to a high stringency of about 0.2 x SSC at 50°C. In addition, the temperature in the wash step can be increased from low stringency conditions at room temperature, about 22°C, to high stringency conditions at about 65°C.

As described in the literature, genes for other hedgehog proteins, e.g., from other animals, can be obtained from mRNA or genomic DNA samples using techniques well known in the art. For example, a cDNA encoding a *hedgehog* protein can be obtained by isolating

total mRNA from a cell, e.g. a mammalian cell, e.g. a human cell, including embryonic cells. Double stranded cDNAs can then be prepared from the total mRNA, and subsequently inserted into a suitable plasmid or bacteriophage vector using any one of a number of known techniques. The gene encoding a *hedgehog* protein can also be cloned using established polymerase chain reaction techniques.

Preferred nucleic acids encode a *hedgehog* polypeptide comprising an amino acid sequence at least 60% homologous or identical, more preferably 70% homologous or identical, and most preferably 80% homologous or identical with an amino acid sequence selected from the group consisting of SEQ ID NOs:8-14. Nucleic acids which encode polypeptides at least about 90%, more preferably at least about 95%, and most preferably at least about 98-99% homology or identity with an amino acid sequence represented in one of SEQ ID NOs:8-14 are also within the scope of the invention.

In addition to native *hedgehog* proteins, h*edgehog* polypeptides preferred by the present invention are at least 60% homologous or identical, more preferably 70% homologous or identical and most preferably 80% homologous or identical with an amino acid sequence represented by any of SEQ ID NOs:8-14. Polypeptides which are at least 90%, more preferably at least 95%, and most preferably at least about 98-99% homologous or identical with a sequence selected from the group consisting of SEQ ID NOs:8-14 are also within the scope of the invention. The only prerequisite is that the *hedgehog* polypeptide is capable of modulating the growth of epithelial cells.

The term "recombinant protein" refers to a polypeptide of the present invention which is produced by recombinant DNA techniques, wherein generally, DNA encoding a *hedgehog* polypeptide is inserted into a suitable expression vector which is in turn used to transform a host cell to produce the heterologous protein. Moreover, the phrase "derived from", with respect to a recombinant *hedgehog* gene, is meant to include within the meaning of "recombinant protein" those proteins having an amino acid sequence of a native *hedgehog* protein, or an amino acid sequence similar thereto which is generated by mutations including substitutions and deletions (including truncation) of a naturally occurring form of the protein.

The method of the present invention can also be carried out using variant forms of the naturally occurring *hedgehog* polypeptides, e.g., mutational variants.

As is known in the art, hedgehog polypeptides can be produced by standard biological techniques or by chemical synthesis. For example, a host cell transfected with a nucleic acid vector directing expression of a nucleotide sequence encoding the subject polypeptides can be cultured under appropriate conditions to allow expression of the peptide to occur. The polypeptide *hedgehog* may be secreted and isolated from a mixture of cells and medium containing the recombinant *hedgehog* polypeptide. Alternatively, the peptide may be retained cytoplasmically by removing the signal peptide sequence from the recombinant *hedgehog* gene and the cells harvested, lysed and the protein isolated. A cell culture includes host cells, media and other byproducts. Suitable media for cell culture are well known in the art. The recombinant *hedgehog* polypeptide can be isolated from cell culture medium, host cells, or both using techniques known in the art for purifying proteins including ion-exchange chromatography, gel filtration chromatography, ultrafiltration, electrophoresis, and immunoaffinity purification with antibodies specific for such peptide. In a preferred embodiment, the recombinant *hedgehog* polypeptide is a fusion protein containing a domain which facilitates its purification, such as an *hedgehog*/GST fusion protein. The host cell may be any prokaryotic or eukaryotic cell.

Recombinant *hedgehog* genes can be produced by ligating nucleic acid encoding an *hedgehog* protein, or a portion thereof, into a vector suitable for expression in either prokaryotic cells, eukaryotic cells, or both. Expression vectors for production of recombinant forms of the subject *hedgehog* polypeptides include plasmids and other vectors. For instance, suitable vectors for the expression of a *hedgehog* polypeptide include plasmids of the types: pBR322-derived plasmids, pEMBL-derived plasmids, pEX-derived plasmids, pBTac-derived plasmids and pUC-derived plasmids for expression in prokaryotic cells, such as *E. coli*.

A number of vectors exist for the expression of recombinant proteins in yeast. For instance, YEP24, YIP5, YEP51, YEP52, pYES2, and YRP17 are cloning and expression vehicles useful in the introduction of genetic constructs into *S. cerevisiae* (see, for example, Broach *et al.* (1983) in *Experimental Manipulation of Gene Expression*, ed. M. Inouye Academic Press, p. 83, incorporated by reference herein). These vectors can replicate in *E. coli* due to the presence of the pBR322 ori, and in *S. cerevisiae* due to the replication determinant of the yeast 2 micron plasmid. In addition, drug resistance markers such as ampicillin can be

used. In an illustrative embodiment, an *hedgehog* polypeptide is produced recombinantly utilizing an expression vector generated by sub-cloning the coding sequence of one of the *hedgehog* genes represented in SEQ ID NOs:1-7.

The preferred mammalian expression vectors contain both prokaryotic sequences, to facilitate the propagation of the vector in bacteria, and one or more eukaryotic transcription units that are expressed in eukaryotic cells. The pcDNAI/amp, pcDNAI/neo, pRc/CMV, pSV2gpt, pSV2neo, pSV2-dhfr, pTk2, pRSVneo, pMSG, pSVT7, pko-neo and pHyg derived vectors are examples of mammalian expression vectors suitable for transfection of eukaryotic cells. Some of these vectors are modified with sequences from bacterial plasmids, such as pBR322, to facilitate replication and drug resistance selection in both prokaryotic and eukaryotic cells. Alternatively, derivatives of viruses such as the bovine papillomavirus (BPV-1), or Epstein-Barr virus (pHEBo, pREP-derived and p205) can be used for transient expression of proteins in eukaryotic cells. The various methods employed in the preparation of the plasmids and transformation of host organisms are well known in the art. For other suitable expression systems for both prokaryotic and eukaryotic cells, as well as general recombinant procedures, see *Molecular Cloning A Laboratory Manual*, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989) Chapters 16 and 17.

In some instances, it may be desirable to express the recombinant *hedgehog* polypeptide by the use of a baculovirus expression system. Examples of such baculovirus expression systems include pVL-derived vectors (such as pVL1392, pVL1393 and pVL941), pAcUW-derived vectors (such as pAcUW1), and pBlueBac-derived vectors (such as the ß-gal containing pBlueBac III).

When it is desirable to express only a portion of an *hedgehog* protein, such as a form lacking a portion of the N-terminus, i.e. a truncation mutant which lacks the signal peptide, it may be necessary to add a start codon (ATG) to the oligonucleotide fragment containing the desired sequence to be expressed. It is well known in the art that a methionine at the N-terminal position can be enzymatically cleaved by the use of the enzyme methionine aminopeptidase (MAP). MAP has been cloned from *E. coli* (Ben-Bassat et al. (1987) *J. Bacteriol.* 169:751-757) and *Salmonella typhimurium* and its *in vitro* activity has been

demonstrated on recombinant proteins (Miller et al. (1987) *PNAS 84*:2718-1722). Therefore, removal of an N-terminal methionine, if desired, can be achieved either *in vivo* by expressing *hedgehog*-derived polypeptides in a host which produces MAP (e.g., *E. coli* or CM89 or *S. cerevisiae*), or *in vitro* by use of purified MAP (e.g., procedure of Miller et al., *supra*).

Alternatively, the coding sequences for the polypeptide can be incorporated as a part of a fusion gene including a nucleotide sequence encoding a different polypeptide. It is widely appreciated that fusion proteins can also facilitate the expression of proteins, and accordingly, can be used in the expression of the *hedgehog* polypeptides of the present invention. For example, *hedgehog* polypeptides can be generated as glutathione-S-transferase (GST-fusion) proteins. Such GST-fusion proteins can enable easy purification of the *hedgehog* polypeptide, as for example by the use of glutathione-derivatized matrices (see, for example, *Current Protocols in Molecular Biology*, eds. Ausubel et al. (N.Y.: John Wiley & Sons, 1991)). In another embodiment, a fusion gene coding for a purification leader sequence, such as a poly-(His)/enterokinase cleavage site sequence, can be used to replace the signal sequence which naturally occurs at the N-terminus of the *hedgehog* protein (e.g.of the pro-form, in order to permit purification of the poly(His)-*hedgehog* protein by affinity chromatography using a Ni^{2+} metal resin. The purification leader sequence can then be subsequently removed by treatment with enterokinase (e.g., see Hochuli et al. (1987) *J. Chromatography* 411:177; and Janknecht et al. *PNAS* 88:8972).

Techniques for making fusion genes are known to those skilled in the art. Essentially, the joining of various DNA fragments coding for different polypeptide sequences is performed in accordance with conventional techniques, employing blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide for appropriate termini, filling-in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and enzymatic ligation. In another embodiment, the fusion gene can be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR amplification of gene fragments can be carried out using anchor primers which give rise to complementary overhangs between two consecutive gene fragments which can subsequently be annealed to generate a chimeric gene sequence (see, for example, *Current Protocols in Molecular Biology*, eds. Ausubel et al. John Wiley & Sons: 1992).

Hedgehog polypeptides may also be chemically modified to create *hedgehog* derivatives by forming covalent or aggregate conjugates with other chemical moieties, such as glycosyl groups, cholesterol, isoprenoids, lipids, phosphate, acetyl groups and the like. Covalent derivatives of *hedgehog* proteins can be prepared by linking the chemical moieties to functional groups on amino acid sidechains of the protein or at the N-terminus or at the C-terminus of the polypeptide.

For instance, *hedgehog* proteins can be generated to include a moiety, other than sequence naturally associated with the protein, that binds a component of the extracellular matrix and enhances localization of the analog to cell surfaces. For example, sequences derived from the fibronectin "type-III repeat", such as a tetrapeptide sequence R-G-D-S (Pierschbacher et al. (1984) *Nature* 309:30-3; and Kornblihtt et al. (1985) *EMBO* 4:1755-9) can be added to the *hedgehog* polypeptide to support attachment of the chimeric molecule to a cell through binding ECM components (Ruoslahti et al. (1987) *Science* 238:491-497; Pierschbacheret al. (1987) *J. Biol. Chem.* 262:17294-8.; Hynes (1987) *Cell* 48:549-54; and Hynes (1992) *Cell* 69:11-25).

In a preferred embodiment, the *hedgehog* polypeptide is isolated from, or is otherwise substantially free of, other cellular proteins, especially other extracellular or cell surface associated proteins which may normally be associated with the *hedgehog* polypeptide, unless provided in the form of fusion protein with the *hedgehog* polypeptide. The term "substantially free of other cellular or extracellular proteins" (also referred to herein as "contaminating proteins") or "substantially pure preparations" or "purified preparations" are defined as encompassing preparations of *hedgehog* polypeptides having less than 20% (by dry weight) contaminating protein, and preferably having less than 5% contaminating protein. By "purified", it is meant that the indicated molecule is present in the substantial absence of other biological macromolecules, such as other proteins. The term "purified" as used herein preferably means at least 80% by dry weight, more preferably in the range of 95-99% by weight, and most preferably at least 99.8% by weight, of biological macromolecules of the same type present (but water, buffers, and other small molecules, especially molecules having a molecular weight of less than 5000, can be present). The term "pure" as used herein preferably has the same numerical limits as "purified" immediately above.

As described above for recombinant polypeptides, isolated *hedgehog* polypeptides can include all or a portion of the amino acid sequences represented in any of SEQ ID NOs:10-18 or 20, or a homologous sequence thereto. Preferred fragments of the subject *hedgehog* proteins correspond to the N-terminal and C-terminal proteolytic fragments of the mature protein. Bioactive fragments of *hedgehog* polypeptides are described in great detail in PCT publications WO 95/18856 and WO 96/17924.

With respect to bioctive fragments of *hedgehog* polypeptide, preferred *hedgehog* therapeutics include at least 50 (contiguous) amino acid residues of a *hedgehog* polypeptide, more preferably at least 100 (contiguous), and even more preferably at least 150 (contiguous) residues.

Another preferred *hedgehog* polypeptide which can be included in the *hedgehog* therapeutic is an N-terminal fragment of the mature protein having a molecular weight of approximately 19 kDa.

Preferred human *hedgehog* proteins include N-terminal fragments corresponding approximately to residues 24-197 of SEQ ID No. 15, 28-202 of SEQ ID No. 16, and 23-198 of SEQ ID No. 17. By "corresponding approximately" it is meant that the sequence of interest is at most 20 amino acid residues different in length to the reference sequence, though more preferably at most 5, 10 or 15 amino acid different in length.

As described above for recombinant polypeptides, isolated *hedgehog* polypeptides can include all or a portion of the amino acid sequences represented in SEQ ID NO:8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, SEQ ID NO:12, SEQ ID NO:13 or SEQ ID NO:14, or a homologous sequence thereto. Preferred fragments of the subject *hedgehog* proteins correspond to the N-terminal and C-terminal proteolytic fragments of the mature protein. Bioactive fragments of hedgehog polypeptides are described in great detail in PCT publications WO 95/18856 and WO 96/17924.

Still other preferred *hedgehog* polypeptides includes an amino acid sequence represented by the formula A-B wherein: (i) A represents all or the portion of the amino acid sequence designated by residues 1-168 of SEQ ID NO:21; and B represents at least one amino acid residue of the amino acid sequence designated by residues 169-221 of SEQ ID NO:21; (ii) A represents all or the portion of the amino acid sequence designated by residues 24-193 of

SEQ ID NO:15; and B represents at least one amino acid residue of the amino acid sequence designated by residues 194-250 of SEQ ID NO:15; (iii) A represents all or the portion of the amino acid sequence designated by residues 25-193 of SEQ ID NO:13; and B represents at least one amino acid residue of the amino acid sequence designated by residues 194-250 of SEQ ID NO:13; (iv) A represents all or the portion of the amino acid sequence designated by residues 23-193 of SEQ ID NO:11; and B represents at least one amino acid residue of the amino acid sequence designated by residues 194-250 of SEQ ID NO:11; (v) A represents all or the portion of the amino acid sequence designated by residues 28-197 of SEQ ID NO:12; and B represents at least one amino acid residue of the amino acid sequence designated by residues 198-250 of SEQ ID NO:12; (vi) A represents all or the portion of the amino acid sequence designated by residues 29-197 of SEQ ID NO:16; and B represents at least one amino acid residue of the amino acid sequence designated by residues 198-250 of SEQ ID NO:16; or (vii) A represents all or the portion of the amino acid sequence designated by residues 23-193 of SEQ ID No. 17, and B represents at least one amino acid residue of the amino acid sequence designated by residues 194-250 of SEQ ID No. 17. In certain preferred embodiments, A and B together represent a contiguous polypeptide sequence designated sequence, A represents at least 25, 50, 75, 100, 125 or 150 (contiguous) amino acids of the designated sequence, and B represents at least 5, 10, or 20 (contiguous) amino acid residues of the amino acid sequence designated by corresponding entry in the sequence listing, and A and B together preferably represent a contiguous sequence corresponding to the sequence listing entry. Similar fragments from other *hedgehog* also contemplated, e.g., fragments which correspond to the preferred fragments from the sequence listing entries which are enumerated above. In preferred embodiments, the *hedgehog* polypeptide includes a C-terminal glycine (or other appropriate residue) which is derivatized with a cholesterol.

Isolated peptidyl portions of *hedgehog* proteins can be obtained by screening peptides recombinantly produced from the corresponding fragment of the nucleic acid encoding such peptides. In addition, fragments can be chemically synthesized using techniques known in the art such as conventional Merrifield solid phase f-Moc or t-Boc chemistry. For example, a *hedgehog* polypeptide of the present invention may be arbitrarily divided into fragments of desired length with no overlap of the fragments, or preferably divided into overlapping fragments of a desired length. The fragments can be produced (recombinantly or by chemical

synthesis) and tested to identify those peptidyl fragments which can function as either agonists or antagonists of a wild-type (e.g., "authentic") *hedgehog* protein. For example, Román et al. (1994) *Eur J Biochem* 222:65-73 describe the use of competitive-binding assays using short, overlapping synthetic peptides from larger proteins to identify binding domains.

The recombinant *hedgehog* polypeptides of the present invention also include homologs of the authentic *hedgehog* proteins, such as versions of those protein which are resistant to proteolytic cleavage, as for example, due to mutations which alter potential cleavage sequences or which inactivate an enzymatic activity associated with the protein. *Hedgehog* homologs of the present invention also include proteins which have been post-translationally modified in a manner different than the authentic protein. Exemplary derivatives of *hedgehog* proteins include polypeptides which lack N-glycosylation sites (e.g. to produce an unglycosylated protein), which lack sites for cholesterolization, and/or which lack N-terminal and/or C-terminal sequences.

Modification of the structure of the subject *hedgehog* polypeptides can also be for such purposes as enhancing therapeutic or prophylactic efficacy, or stability (e.g., *ex vivo* shelf life and resistance to proteolytic degradation *in vivo*). Such modified peptides, when designed to retain at least one activity of the naturally-occurring form of the protein, are considered functional equivalents of the *hedgehog* polypeptides described in more detail herein. Such modified peptides can be produced, for instance, by amino acid substitution, deletion, or addition.

It is well known in the art that one could reasonably expect that certain isolated replacements of amino acids, e.g., replacement of an amino acid residue with another related amino acid (i.e. isosteric and/or isoelectric mutations), can be carried out without major effect on the biological activity of the resulting molecule. Conservative replacements are those that take place within a family of amino acids that are related in their side chains. Genetically encoded amino acids are can be divided into four families: (1) acidic = aspartate, glutamate; (2) basic = lysine, arginine, histidine; (3) nonpolar = alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan; and (4) uncharged polar = glycine, asparagine, glutamine, cysteine, serine, threonine, tyrosine. Phenylalanine, tryptophan, and tyrosine are sometimes classified jointly as aromatic amino acids. In similar fashion, the amino acid

repertoire can be grouped as (1) acidic = aspartate, glutamate; (2) basic = lysine, arginine histidine, (3) aliphatic = glycine, alanine, valine, leucine, isoleucine, serine, threonine, with serine and threonine optionally be grouped separately as aliphatic-hydroxyl; (4) aromatic = phenylalanine, tyrosine, tryptophan; (5) amide = asparagine, glutamine; and (6) sulfur - containing = cysteine and methionine. (see, for example, *Biochemistry*, 2nd ed., Ed. by L. Stryer, WH Freeman and Co.: 1981). Whether a change in the amino acid sequence of a peptide results in a functional *hedgehog* homolog (e.g. functional in the sense that it acts to mimic or antagonize the wild-type form) can be readily determined by assessing the ability of the variant peptide to produce a response in cells in a fashion similar to the wild-type protein, or competitively inhibit such a response. Polypeptides in which more than one replacement has taken place can readily be tested in the same manner.

It is specifically contemplated that the methods of the present invention can be carried using homologs of naturally occurring hedgehog proteins. In one embodiment, the invention contemplates using hedgehog polypeptides generated by combinatorial mutagenesis. Such methods, as are known in the art, are convenient for generating both point and truncation mutants, and can be especially useful for identifying potential variant sequences (e.g. homologs) that are functional in binding to a receptor for *hedgehog* proteins. The purpose of screening such combinatorial libraries is to generate, for example, novel *hedgehog* homologs which can act as either agonists or antagonist. To illustrate, *hedgehog* homologs can be engineered by the present method to provide more efficient binding to a cognate receptor, such as *patched*, yet still retain at least a portion of an activity associated with *hedgehog*. Thus, combinatorially-derived homologs can be generated to have an increased potency relative to a naturally occurring form of the protein. Likewise, *hedgehog* homologs can be generated by the present combinatorial approach to act as antagonists, in that they are able to mimic, for example, binding to other extracellular matrix components (such as receptors), yet not induce any biological response, thereby inhibiting the action of authentic *hedgehog* or *hedgehog* agonists. Moreover, manipulation of certain domains of *hedgehog* by the present method can provide domains more suitable for use in fusion proteins, such as one that incorporates portions of other proteins which are derived from the extracellular matrix and/or which bind extracellular matrix components.

To further illustrate the state of the art of combinatorial mutagenesis, it is noted that the review article of Gallop et al. (1994) *J Med Chem* 37:1233 describes the general state of the art of combinatorial libraries as of the earlier 1990's. In particular, Gallop et al state at page 1239 "[s]creening the analog libraries aids in determining the minimum size of the active sequence and in identifying those residues critical for binding and intolerant of substitution". In addition, the Ladner et al. PCT publication WO90/02809, the Goeddel et al. U.S. Patent 5,223,408, and the Markland et al. PCT publication WO92/15679 illustrate specific techniques which one skilled in the art could utilize to generate libraries of *hedgehog* variants which can be rapidly screened to identify variants/fragments which retained a particular activity of the *hedgehog* polypeptides. These techniques are exemplary of the art and demonstrate that large libraries of related variants/truncants can be generated and assayed to isolate particular variants without undue experimentation. Gustin et al. (1993) *Virology* 193:653, and Bass et al. (1990) *Proteins: Structure, Function and Genetics* 8:309-314 also describe other exemplary techniques from the art which can be adapted as means for generating mutagenic variants of *hedgehog* polypeptides.

Indeed, it is plain from the combinatorial mutagenesis art that large scale mutagenesis of hedgehog proteins, without any preconceived ideas of which residues were critical to the biological function, and generate wide arrays of variants having equivalent biological activity. Indeed, it is the ability of combinatorial techniques to screen billions of different variants by high throughout analysis that removes any requirement of *a priori* understanding or knowledge of critical residues.

To illsutrate, the amino acid sequences for a population of *hedgehog* homologs or other related proteins are aligned, preferably to promote the highest homology possible. Such a population of variants can include, for example, *hedgehog* homologs from one or more species. Amino acids which appear at each position of the aligned sequences are selected to create a degenerate set of combinatorial sequences. In a preferred embodiment, the variegated library of *hedgehog* variants is generated by combinatorial mutagenesis at the nucleic acid level, and is encoded by a variegated gene library. For instance, a mixture of synthetic oligonucleotides can be enzymatically ligated into gene sequences such that the degenerate set of potential *hedgehog* sequences are expressible as individual polypeptides, or alternatively, as

a set of larger fusion proteins (e.g. for phage display) containing the set of *hedgehog* sequences therein.

As illustrated in PCT publication WO 95/18856, to analyze the sequences of a population of variants, the amino acid sequences of interest can be aligned relative to sequence homology. The presence or absence of amino acids from an aligned sequence of a particular variant is relative to a chosen consensus length of a reference sequence, which can be real or artificial.

In an illustrative embodiment, alignment of exons 1, 2 and a portion of exon 3 encoded sequences (e.g. the N-terminal approximately 221 residues of the mature protein) of each of the *Shh* clones produces a degenerate set of *Shh* polypeptides represented by the general formula:

C-G-P-G-R-G-X(1)-G-X(2)-R-R-H-P-K-K-L-T-P-L-A-Y-K-Q-F-I-P-N-V-A-E-K-T-L-G-A-S-G-R-Y-E-G-K-I-X(3)-R-N-S-E-R-F-K-E-L-T-P-N-Y-N-P-D-I-I-F-K-D-E-E-N-T-G-A-D-R-L-M-T-Q-R-C-K-D-K-L-N-X(4)-L-A-I-S-V-M-N-X(5)-W-P-G-V-X(6)-L-R-V-T-E-G-W-D-E-D-G-H-H-X(7)-E-E-S-L-H-Y-E-G-R-A-V-D-I-T-T-S-D-R-D-X(8)-S-K-Y-G-X(9)-L-X(10)-R-L-A-V-E-A-G-F-D-W-V-Y-Y-E-S-K-A-H-I-H-C-S-V-K-A-E-N-S-V-A-A-K-S-G-G-C-F-P-G-S-A-X(11)-V-X(12)-L-X(13)-X(14)-G-G-X(15)-K-X-(16)-V-K-D-L-X(17)-P-G-D-X(18)-V-L-A-A-D-X(19)-X(20)-G-X(21)-L-X(22)-X(23)-S-D-F-X(24)-X(25)-F-X(26)-D-R (SEQ ID NO: 21

wherein each of the degenerate positions "X" can be an amino acid which occurs in that position in one of the human, mouse, chicken or zebrafish *Shh* clones, or, to expand the library, each X can also be selected from amongst amino acid residue which would be conservative substitutions for the amino acids which appear naturally in each of those positions. For instance, Xaa(1) represents Gly, Ala, Val, Leu, Ile, Phe, Tyr or Trp ; Xaa(2) represents Arg, His or Lys; Xaa(3) represents Gly, Ala, Val, Leu, Ile, Ser or Thr; Xaa(4) represents Gly, Ala, Val, Leu, Ile, Ser or Thr; Xaa(5) represents Lys, Arg, His, Asn or Gln; Xaa(6) represents Lys, Arg or His; Xaa(7) represents Ser, Thr, Tyr, Trp or Phe; Xaa(8) represents Lys, Arg or His; Xaa(9) represents Met, Cys, Ser or Thr; Xaa(10) represents Gly, Ala, Val, Leu, Ile, Ser or Thr; Xaa(11) represents Leu, Val, Met, Thr or Ser; Xaa(12) represents His, Phe, Tyr, Ser, Thr, Met or Cys; Xaa(13) represents Gln, Asn, Glu, or Asp;

Xaa(14) represents His, Phe, Tyr, Thr, Gln, Asn, Glu or Asp; Xaa(15) represents Gln, Asn, Glu, Asp, Thr, Ser, Met or Cys; Xaa(16) represents Ala, Gly, Cys, Leu, Val or Met; Xaa(17) represents Arg, Lys, Met, Ile, Asn, Asp, Glu, Gln, Ser, Thr or Cys; Xaa(18) represents Arg, Lys, Met or Ile; Xaa(19) represents Ala, Gly, Cys, Asp, Glu, Gln, Asn, Ser, Thr or Met; Xaa(20) represents Ala, Gly, Cys, Asp, Asn, Glu or Gln; Xaa(21) represents Arg, Lys, Met, Ile, Asn, Asp, Glu or Gln; Xaa(22) represent Leu, Val, Met or Ile; Xaa(23) represents Phe, Tyr, Thr, His or Trp; Xaa(24) represents Ile, Val, Leu or Met; Xaa(25) represents Met, Cys, Ile, Leu, Val, Thr or Ser; Xaa(26) represents Leu, Val, Met, Thr or Ser. In an even more expansive library, each X can be selected from any amino acid.

In similar fashion, alignment of each of the human, mouse, chicken and zebrafish *hedgehog* clones, can provide a degenerate polypeptide sequence represented by the general formula:

C-G-P-G-R-G-X(1)-X(2)-X(3)-R-R-X(4)-X(5)-X(6)-P-K-X(7)-L-X(8)-P-L-X(9)-Y-K-Q-F-X(10)-P-X(11)-X(12)-X(13)-E-X(14)-T-L-G-A-S-G-X(15)-X(16)-E-G-X(17)-X(18)-X(19)-R-X(20)-S-E-R-F-X(21)-X(22)-L-T-P-N-Y-N-P-D-I-I-F-K-D-E-E-N-X(23)-G-A-D-R-L-M-T-X(24)-R-C-K-X(25)-X(26)-X(27)-N-X(28)-L-A-I-S-V-M-N-X(29)-W-P-G-V-X(30)-L-R-V-T-E-G-X(31)-D-E-D-G-H-H-X(32)-X(33)-X(34)-S-L-H-Y-E-G-R-A-X(35)-D-I-T-T-S-D-R-D-X(36)-X(37)-K-Y-G-X(38)-L-X(39)-R-L-A-V-E-A-G-F-D-W-V-Y-Y-E-S-X(40)-X(41)-H-X(42)-H-X(43)-S-V-K-X(44)-X(45) (SEQ IDNo:22)

wherein, as above, each of the degenerate positions "X" can be an amino acid which occurs in a corresponding position in one of the wild-type clones, and may also include amino acid residue which would be conservative substitutions, or each X can be any amino acid residue. In an exemplary embodiment, Xaa(1) represents Gly, Ala, Val, Leu, Ile, Pro, Phe or Tyr; Xaa(2) represents Gly, Ala, Val, Leu or Ile; Xaa(3) represents Gly, Ala, Val, Leu, Ile, Lys, His or Arg; Xaa(4) represents Lys, Arg or His; Xaa(5) represents Phe, Trp, Tyr or an amino acid gap; Xaa(6) represents Gly, Ala, Val, Leu, Ile or an amino acid gap; Xaa(7) represents Asn, Gln, His, Arg or Lys; Xaa(8) represents Gly, Ala, Val, Leu, Ile, Ser or Thr; Xaa(9) represents Gly, Ala, Val, Leu, Ile, Ser or Thr; Xaa(10) represents Gly, Ala, Val, Leu, Ile, Ser or Thr; Xaa(11) represents Ser, Thr, Gln or Asn; Xaa(12) represents Met, Cys, Gly, Ala, Val, Leu, Ile, Ser or Thr; Xaa(13) represents Gly, Ala, Val, Leu, Ile or Pro; Xaa(14) represents Arg, His or Lys; Xaa(15) represents Gly, Ala, Val, Leu, Ile, Pro, Arg, His or Lys; Xaa(16) represents Gly,

Ala, Val, Leu, Ile, Phe or Tyr; Xaa(17) represents Arg, His or Lys; Xaa(18) represents Gly, Ala, Val, Leu, Ile, Ser or Thr; Xaa(19) represents Thr or Ser; Xaa(20) represents Gly, Ala, Val, Leu, Ile, Asn or Gln; Xaa(21) represents Arg, His or Lys; Xaa(22) represents Asp or Glu; Xaa(23) represents Ser or Thr; Xaa(24) represents Glu, Asp, Gln or Asn; Xaa(25) represents Glu or Asp; Xaa(26) represents Arg, His or Lys; Xaa(27) represents Gly, Ala, Val, Leu or Ile; Xaa(28) represents Gly, Ala, Val, Leu, Ile, Thr or Ser; Xaa(29) represents Met, Cys, Gln, Asn, Arg, Lys or His; Xaa(30) represents Arg, His or Lys; Xaa(31) represents Trp, Phe, Tyr, Arg, His or Lys; Xaa(32) represents Gly, Ala, Val, Leu, Ile, Ser, Thr, Tyr or Phe; Xaa(33) represents Gln, Asn, Asp or Glu; Xaa(34) represents Asp or Glu; Xaa(35) represents Gly, Ala, Val, Leu, or Ile; Xaa(36) represents Arg, His or Lys; Xaa(37) represents Asn, Gln, Thr or Ser; Xaa(38) represents Gly, Ala, Val, Leu, Ile, Ser, Thr, Met or Cys; Xaa(39) represents Gly, Ala, Val, Leu, Ile, Thr or Ser; Xaa(40) represents Arg, His or Lys; Xaa(41) represents Asn, Gln, Gly, Ala, Val, Leu or Ile; Xaa(42) represents Gly, Ala, Val, Leu or Ile; Xaa(43) represents Gly, Ala, Val, Leu, Ile, Ser, Thr or Cys; Xaa(44) represents Gly, Ala, Val, Leu, Ile, Thr or Ser; and Xaa(45) represents Asp or Glu.

There are many ways by which the library of potential *hedgehog* homologs can be generated from a degenerate oligonucleotide sequence. Chemical synthesis of a degenerate gene sequence can be carried out in an automatic DNA synthesizer, and the synthetic genes then ligated into an appropriate expression vector. The purpose of a degenerate set of genes is to provide, in one mixture, all of the sequences encoding the desired set of potential *hedgehog* sequences. The synthesis of degenerate oligonucleotides is well known in the art (see for example, Narang, SA (1983) *Tetrahedron* 39:3; Itakura et al. (1981) *Recombinant DNA, Proc 3rd Cleveland Sympos. Macromolecules*, ed. AG Walton, Amsterdam: Elsevier pp273-289; Itakura et al. (1984) *Annu. Rev. Biochem.* 53:323; Itakura et al. (1984) *Science* 198:1056; Ike et al. (1983) *Nucleic Acid Res.* 11:477. Such techniques have been employed in the directed evolution of other proteins (see, for example, Scott et al. (1990) *Science* 249:386-390; Roberts et al. (1992) *PNAS* 89:2429-2433; Devlin et al. (1990) *Science* 249: 404-406; Cwirla et al. (1990) *PNAS* 87: 6378-6382; as well as U.S. Patents Nos. 5,223,409, 5,198,346, and 5,096,815).

A wide range of techniques are known in the art for screening gene products of combinatorial libraries made by point mutations, and for screening cDNA libraries for gene products having a certain property. Such techniques will be generally adaptable for rapid screening of the gene libraries generated by the combinatorial mutagenesis of *hedgehog* homologs. The most widely used techniques for screening large gene libraries typically comprises cloning the gene library into replicable expression vectors, transforming appropriate cells with the resulting library of vectors, and expressing the combinatorial genes under conditions in which detection of a desired activity facilitates relatively easy isolation of the vector encoding the gene whose product was detected. Each of the illustrative assays described below are amenable to high through-put analysis as necessary to screen large numbers of degenerate *hedgehog* sequences created by combinatorial mutagenesis techniques.

In one embodiment, the combinatorial library is designed to be secreted (e.g. the polypeptides of the library all include a signal sequence but no transmembrane or cytoplasmic domains), and is used to transfect a eukaryotic cell that can be co-cultured with epithelial stem cells. A functional *hedgehog* protein secreted by the cells expressing the combinatorial library will diffuse to neighboring epithelial cells and induce a particular biological response, such as proliferation. The pattern of detection of proliferation will resemble a gradient function, and will allow the isolation (generally after several repetitive rounds of selection) of cells producing *hedgehog* homologs active as proliferative agents with respect to epithelial cells. Likewise, *hedgehog* antagonists can be selected in similar fashion by the ability of the cell producing a functional antagonist to protect neighboring cells (e.g., to inhibit proliferation) from the effect of wild-type *hedgehog* added to the culture media.

To illustrate, target epithelial cells are cultured in 24-well microtitre plates. Other eukaryotic cells are transfected with the combinatorial *hedgehog* gene library and cultured in cell culture inserts (e.g. Collaborative Biomedical Products, Catalog #40446) that are able to fit into the wells of the microtitre plate. The cell culture inserts are placed in the wells such that recombinant *hedgehog* homologs secreted by the cells in the insert can diffuse through the porous bottom of the insert and contact the target cells in the microtitre plate wells. After a period of time sufficient for functional forms of a *hedgehog* protein to produce a measurable response in the target cells, such as proliferation, the inserts are removed and the effect of the

variant *hedgehog* proteins on the target cells determined. Cells from the inserts corresponding to wells which score positive for activity can be split and re-cultured on several inserts, the process being repeated until the active clones are identified.

In yet another screening assay, the candidate *hedgehog* gene products are displayed on the surface of a cell or viral particle, and the ability of particular cells or viral particles to associate with a *hedgehog*-binding moiety (such as the *patched* protein or other *hedgehog* receptor) via this gene product is detected in a "panning assay". Such panning steps can be carried out on cells cultured from embryos. For instance, the gene library can be cloned into the gene for a surface membrane protein of a bacterial cell, and the resulting fusion protein detected by panning (Ladner et al., WO 88/06630; Fuchs et al. (1991) *Bio/Technology* 9:1370-1371; and Goward et al. (1992) *TIBS* 18:136-140). In a similar fashion, fluorescently labeled molecules which bind *hedgehog* can be used to score for potentially functional *hedgehog* homologs. Cells can be visually inspected and separated under a fluorescence microscope, or, where the morphology of the cell permits, separated by a fluorescence-activated cell sorter.

In an alternate embodiment, the gene library is expressed as a fusion protein on the surface of a viral particle. For instance, in the filamentous phage system, foreign peptide sequences can be expressed on the surface of infectious phage, thereby conferring two significant benefits. First, since these phage can be applied to affinity matrices at very high concentrations, large number of phage can be screened at one time. Second, since each infectious phage displays the combinatorial gene product on its surface, if a particular phage is recovered from an affinity matrix in low yield, the phage can be amplified by another round of infection. The group of almost identical *E.coli* filamentous phages M13, fd, and f1 are most often used in phage display libraries, as either of the phage gIII or gVIII coat proteins can be used to generate fusion proteins without disrupting the ultimate packaging of the viral particle (Ladner et al. PCT publication WO 90/02909; Garrard et al., PCT publication WO 92/09690; Marks et al. (1992) J. Biol. Chem. 267:16007-16010; Griffths et al. (1993) EMBO J 12:725-734; Clackson et al. (1991) Nature 352:624-628; and Barbas et al. (1992) PNAS 89:4457-4461).

In an illustrative embodiment, the recombinant phage antibody system (RPAS, Pharamacia Catalog number 27-9400-01) can be easily modified for use in expressing and

screening *hedgehog* combinatorial libraries. For instance, the pCANTAB 5 phagemid of the RPAS kit contains the gene which encodes the phage gIII coat protein. The *hedgehog* combinatorial gene library can be cloned into the phagemid adjacent to the gIII signal sequence such that it will be expressed as a gIII fusion protein. After ligation, the phagemid is used to transform competent *E. coli* TG1 cells. Transformed cells are subsequently infected with M13KO7 helper phage to rescue the phagemid and its candidate *hedgehog* gene insert. The resulting recombinant phage contain phagemid DNA encoding a specific candidate *hedgehog*, and display one or more copies of the corresponding fusion coat protein. The phage-displayed candidate *hedgehog* proteins which are capable of binding an *hedgehog* receptor are selected or enriched by panning. For instance, the phage library can be applied to cells which express the *patched* protein and unbound phage washed away from the cells. The bound phage is then isolated, and if the recombinant phage express at least one copy of the wild type gIII coat protein, they will retain their ability to infect *E. coli*. Thus, successive rounds of reinfection of *E. coli*, and panning will greatly enrich for *hedgehog* homologs, which can then be screened for further biological activities in order to differentiate agonists and antagonists.

Combinatorial mutagenesis has a potential to generate very large libraries of mutant proteins, e.g., in the order of 10^{26} molecules. Combinatorial libraries of this size may be technically challenging to screen even with high throughput screening assays such as phage display. To overcome this problem, a new technique has been developed recently, recursive ensemble mutagenesis (REM), which allows one to avoid the very high proportion of non-functional proteins in a random library and simply enhances the frequency of functional proteins, thus decreasing the complexity required to achieve a useful sampling of sequence space. REM is an algorithm which enhances the frequency of functional mutants in a library when an appropriate selection or screening method is employed (Arkin and Yourvan, 1992, *PNAS USA* 89:7811-7815; Yourvan et al., 1992, *Parallel Problem Solving from Nature*, 2., In Maenner and Manderick, eds., Elsevir Publishing Co., Amsterdam, pp. 401-410; Delgrave et al., 1993, *Protein Engineering* 6(3):327-331).

The invention also provides for reduction of the *hedgehog* protein to generate mimetics, e.g. peptide or non-peptide agents, which are able to disrupt binding of a *hedgehog* polypeptide of the present invention with an *hedgehog* receptor. Thus, such mutagenic

techniques as described above are also useful to map the determinants of the *hedgehog* proteins which participate in protein-protein interactions involved in, for example, binding of the subject *hedgehog* polypeptide to other extracellular matrix components. To illustrate, the critical residues of a subject *hedgehog* polypeptide which are involved in molecular recognition of an *hedgehog* receptor such as *patched* can be determined and used to generate *hedgehog*-derived peptidomimetics which competitively inhibit binding of the authentic *hedgehog* protein with that moiety. By employing, for example, scanning mutagenesis to map the amino acid residues of each of the subject *hedgehog* proteins which are involved in binding other extracellular proteins, peptidomimetic compounds can be generated which mimic those residues of the *hedgehog* protein which facilitate the interaction. Such mimetics may then be used to interfere with the normal function of a *hedgehog* protein. For instance, non-hydrolyzable peptide analogs of such residues can be generated using benzodiazepine (e.g., see Freidinger et al. in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), azepine (e.g., see Huffman et al. in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), substituted gama lactam rings (Garvey et al. in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), keto-methylene pseudopeptides (Ewenson et al. (1986) *J Med Chem* 29:295; and Ewenson et al. in *Peptides: Structure and Function* (Proceedings of the 9th American Peptide Symposium) Pierce Chemical Co. Rockland, IL, 1985), β-turn dipeptide cores (Nagai et al. (1985) *Tetrahedron Lett* 26:647; and Sato et al. (1986) *J Chem Soc Perkin Trans* 1:1231), and β-aminoalcohols (Gordon et al. (1985) *Biochem Biophys Res Commun*126:419; and Dann et al. (1986) *Biochem Biophys Res Commun* 134:71).

Recombinantly produced forms of the hedgehog proteins can be produced using, e.g, expression vectors containing a nucleic acid encoding a *hedgehog* polypeptide, operably linked to at least one transcriptional regulatory sequence. Operably linked is intended to mean that the nucleotide sequence is linked to a regulatory sequence in a manner which allows expression of the nucleotide sequence. Regulatory sequences are art-recognized and are selected to direct expression of a *hedgehog* polypeptide. Accordingly, the term transcriptional regulatory sequence includes promoters, enhancers and other expression control elements. Such regulatory sequences are described in Goeddel; *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1990). For instance, any of a wide

variety of expression control sequences, sequences that control the expression of a DNA sequence when operatively linked to it, may be used in these vectors to express DNA sequences encoding *hedgehog* polypeptide. Such useful expression control sequences, include, for example, a viral LTR, such as the LTR of the Moloney murine leukemia virus, the early and late promoters of SV40, adenovirus or cytomegalovirus immediate early promoter, the lac system, the trp system, the TAC or TRC system, T7 promoter whose expression is directed by T7 RNA polymerase, the major operator and promoter regions of phage λ , the control regions for fd coat protein, the promoter for 3-phosphoglycerate kinase or other glycolytic enzymes, the promoters of acid phosphatase, e.g., Pho5, the promoters of the yeast α-mating factors, the polyhedron promoter of the baculovirus system and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof. It should be understood that the design of the expression vector may depend on such factors as the choice of the host cell to be transformed and/or the type of protein desired to be expressed. Moreover, the vector's copy number, the ability to control that copy number and the expression of any other proteins encoded by the vector, such as antibiotic markers, should also be considered.

In addition to providing a ready source of hedgehog polypeptides for purification, the gene constructs of the present invention can also be used as a part of a gene therapy protocol to deliver nucleic acids encoding either an agonistic or antagonistic form of a *hedgehog* polypeptide. Thus, another aspect of the invention features expression vectors for *in vivo* transfection of a *hedgehog* polypeptide in particular cell types so as cause ectopic expression of a *hedgehog* polypeptide in an epithelial tissue.

Formulations of such expression constructs may be administered in any biologically effective carrier, e.g. any formulation or composition capable of effectively delivering the recombinant gene to cells *in vivo*. Approaches include insertion of the hedgehog coding sequence in viral vectors including recombinant retroviruses, adenovirus, adeno-associated virus, and herpes simplex virus-1, or recombinant bacterial or eukaryotic plasmids. Viral vectors transfect cells directly; plasmid DNA can be delivered with the help of, for example, cationic liposomes (lipofectin) or derivatized (e.g. antibody conjugated), polylysine conjugates, gramacidin S, artificial viral envelopes or other such intracellular carriers, as well

as direct injection of the gene construct or $CaPO_4$ precipitation carried out *in vivo*. It will be appreciated that because transduction of appropriate target cells represents the critical first step in gene therapy, choice of the particular gene delivery system will depend on such factors as the phenotype of the intended target and the route of administration, e.g. locally or systemically. Furthermore, it will be recognized that the particular gene construct provided for *in vivo* transduction of *hedgehog* expression are also useful for *in vitro* transduction of cells, such as for use in the *ex vivo* tissue culture systems described below.

A preferred approach for *in vivo* introduction of nucleic acid into a cell is by use of a viral vector containing nucleic acid, e.g. a cDNA, encoding the particular form of the *hedgehog* polypeptide desired. Infection of cells with a viral vector has the advantage that a large proportion of the targeted cells can receive the nucleic acid. Additionally, molecules encoded within the viral vector, e.g., by a cDNA contained in the viral vector, are expressed efficiently in cells which have taken up viral vector nucleic acid.

Retrovirus vectors and adeno-associated virus vectors are generally understood to be the recombinant gene delivery system of choice for the transfer of exogenous genes *in vivo*, particularly into humans. These vectors provide efficient delivery of genes into cells, and the transferred nucleic acids are stably integrated into the chromosomal DNA of the host. A major prerequisite for the use of retroviruses is to ensure the safety of their use, particularly with regard to the possibility of the spread of wild-type virus in the cell population. The development of specialized cell lines (termed "packaging cells") which produce only replication-defective retroviruses has increased the utility of retroviruses for gene therapy, and defective retroviruses are well characterized for use in gene transfer for gene therapy purposes (for a review see Miller, A.D. (1990) *Blood* 76:271). Thus, recombinant retrovirus can be constructed in which part of the retroviral coding sequence (*gag, pol, env*) has been replaced by nucleic acid encoding a hedgehog polypeptide and renders the retrovirus replication defective. The replication defective retrovirus is then packaged into virions which can be used to infect a target cell through the use of a helper virus by standard techniques. Protocols for producing recombinant retroviruses and for infecting cells *in vitro* or *in vivo* with such viruses can be found in Current Protocols in Molecular Biology, Ausubel, F.M. et al. (eds.) Greene Publishing Associates, (1989), Sections 9.10-9.14 and other standard laboratory manuals.

Examples of suitable retroviruses include pLJ, pZIP, pWE and pEM which are well known to those skilled in the art. Examples of suitable packaging virus lines for preparing both ecotropic and amphotropic retroviral systems include Crip, Cre, 2 and Am. Retroviruses have been used to introduce a variety of genes into many different cell types, including epithelial cells, *in vitro* and/or *in vivo* (see for example Eglitis, et al. (1985) *Science* 230:1395-1398; Danos and Mulligan (1988) *Proc. Natl. Acad. Sci. USA* 85:6460-6464; Wilson et al. (1988) *Proc. Natl. Acad. Sci. USA* 85:3014-3018; Armentano et al. (1990) *Proc. Natl. Acad. Sci. USA* 87:6141-6145; Huber et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:8039-8043; Ferry et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:8377-8381; Chowdhury et al. (1991) *Science* 254:1802-1805; van Beusechem et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:7640-7644; Kay et al. (1992) *Human Gene Therapy* 3:641-647; Dai et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:10892-10895; Hwu et al. (1993) *J. Immunol.* 150:4104-4115; U.S. Patent No. 4,868,116; U.S. Patent No. 4,980,286; PCT Application WO 89/07136; PCT Application WO 89/02468; PCT Application WO 89/05345; and PCT Application WO 92/07573).

Furthermore, it has been shown that it is possible to limit the infection spectrum of retroviruses and consequently of retroviral-based vectors, by modifying the viral packaging proteins on the surface of the viral particle (see, for example PCT publications WO93/25234 and WO94/06920). For instance, strategies for the modification of the infection spectrum of retroviral vectors include: coupling antibodies specific for cell surface antigens to the viral *env* protein (Roux et al. (1989) *PNAS* 86:9079-9083; Julan et al. (1992) *J. Gen Virol* 73:3251-3255; and Goud et al. (1983) *Virology* 163:251-254); or coupling cell surface receptor ligands to the viral *env* proteins (Neda et al. (1991) *J Biol Chem* 266:14143-14146). Coupling can be in the form of the chemical cross-linking with a protein or other variety (e.g. lactose to convert the *env* protein to an asialoglycoprotein), as well as by generating fusion proteins (e.g. single-chain antibody/*env* fusion proteins). This technique, while useful to limit or otherwise direct the infection to certain tissue types, can also be used to convert an ecotropic vector in to an amphotropic vector.

Moreover, use of retroviral gene delivery can be further enhanced by the use of tissue- or cell-specific transcriptional regulatory sequences which control expression of the *hedgehog* gene of the retroviral vector.

Another viral gene delivery system useful in the present method utilizes adenovirus-derived vectors. The genome of an adenovirus can be manipulated such that it encodes and expresses a gene product of interest but is inactivated in terms of its ability to replicate in a normal lytic viral life cycle. See for example Berkner et al. (1988) *BioTechniques* 6:616; Rosenfeld et al. (1991) *Science* 252:431-434; and Rosenfeld et al. (1992) *Cell* 68:143-155. Suitable adenoviral vectors derived from the adenovirus strain Ad type 5 dl324 or other strains of adenovirus (e.g., Ad2, Ad3, Ad7 etc.) are well known to those skilled in the art. Recombinant adenoviruses can be advantageous in certain circumstances in that they can be used to infect a wide variety of cell types, including epithelial cells (Rosenfeld et al. (1992) cited *supra*). Furthermore, the virus particle is relatively stable and amenable to purification and concentration, and as above, can be modified so as to affect the spectrum of infectivity. Additionally, introduced adenoviral DNA (and foreign DNA contained therein) is not integrated into the genome of a host cell but remains episomal, thereby avoiding potential problems that can occur as a result of insertional mutagenesis in situations where introduced DNA becomes integrated into the host genome (e.g., retroviral DNA). Moreover, the carrying capacity of the adenoviral genome for foreign DNA is large (up to 8 kilobases) relative to other gene delivery vectors (Berkner et al. cited *supra*; Haj-Ahmand and Graham (1986) *J. Virol.* 57:267). Most replication-defective adenoviral vectors currently in use and therefore favored by the present invention are deleted for all or parts of the viral E1 and E3 genes but retain as much as 80% of the adenoviral genetic material (see, e.g., Jones et al. (1979) *Cell* 16:683; Berkner et al., *supra*; and Graham et al. in Methods in Molecular Biology, E.J. Murray, Ed. (Humana, Clifton, NJ, 1991) vol. 7. pp. 109-127). Expression of the inserted *hedgehog* gene can be under control of, for example, the E1A promoter, the major late promoter (MLP) and associated leader sequences, the E3 promoter, or exogenously added promoter sequences.

In addition to viral transfer methods, such as those illustrated above, non-viral methods can also be employed to cause expression of a *hedgehog* polypeptide in the tissue of an animal. Most nonviral methods of gene transfer rely on normal mechanisms used by mammalian cells for the uptake and intracellular transport of macromolecules. In preferred embodiments, non-viral gene delivery systems of the present invention rely on endocytic pathways for the uptake of the *hedgehog* polypeptide gene by the targeted cell. Exemplary gene delivery systems of

this type include liposomal derived systems, poly-lysine conjugates, and artificial viral envelopes.

In clinical settings, the gene delivery systems for the therapeutic *hedgehog* gene can be introduced into a patient by any of a number of methods, each of which is familiar in the art. For instance, a pharmaceutical preparation of the gene delivery system can be introduced systemically, e.g. by intravenous injection, and specific transduction of the protein in the target cells occurs predominantly from specificity of transfection provided by the gene delivery vehicle, cell-type or tissue-type expression due to the transcriptional regulatory sequences controlling expression of the receptor gene, or a combination thereof. In other embodiments, initial delivery of the recombinant gene is more limited with introduction into the animal being quite localized. For example, the gene delivery vehicle can be introduced by catheter (see U.S. Patent 5,328,470) or by stereotactic injection (e.g. Chen et al. (1994) *PNAS* 91: 3054-3057). A *hedgehog* expression construct can be delivered in a gene therapy construct to dermal cells by, e.g., electroporation using techniques described, for example, by Dev et al. ((1994) *Cancer Treat Rev* 20:105-115).

The pharmaceutical preparation of the gene therapy construct can consist essentially of the gene delivery system in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery system can be produced intact from recombinant cells, e.g. retroviral vectors, the pharmaceutical preparation can comprise one or more cells which produce the gene delivery system.

In yet another embodiment, the hedgehog or ptc therapeutic can be a "gene activation" construct which, by homologous recombination with a genomic DNA, alters the transcriptional regulatory sequences of an endogenous gene. For instance, the gene activation construct can replace the endogenous promoter of a *hedgehog* gene with a heterologous promoter, e.g., one which causes consitutive expression of the *hedgehog* gene or which causes inducible expression of the gene under conditions different from the normal expression pattern of the gene. Other genes in the *patched* signaling pathway can be similarly targeted. A vareity of different formats for the gene activation constructs are available. See, for example, the

Transkaryotic Therapies, Inc PCT publications WO93/09222, WO95/31560, WO96/29411, WO95/31560 and WO94/12650.

In preferred embodiments, the nucleotide sequence used as the gene activation construct can be comprised of (1) DNA from some portion of the endogenous *hedgehog* gene (exon sequence, intron sequence, promoter sequences, etc.) which direct recombination and (2) heterologous transcriptional regulatory sequence(s) which is to be operably linked to the coding sequence for the genomic *hedgehog* gene upon recombination of the gene activation construct. For use in generating cultures of *hedgehog* producing cells, the construct may further include a reporter gene to detect the presence of the knockout construct in the cell.

The gene activation construct is inserted into a cell, and integrates with the genomic DNA of the cell in such a position so as to provide the heterologous regulatory sequences in operative association with the native *hedgehog* gene. Such insertion occurs by homologous recombination, i.e., recombination regions of the activation construct that are homologous to the endogenous *hedgehog* gene sequence hybridize to the genomic DNA and recombine with the genomic sequences so that the construct is incorporated into the corresponding position of the genomic DNA.

The terms "recombination region" or "targeting sequence" refer to a segment (i.e., a portion) of a gene activation construct having a sequence that is substantially identical to or substantially complementary to a genomic gene sequence, e.g., including 5' flanking sequences of the genomic gene, and can facilitate homologous recombination between the genomic sequence and the targeting transgene construct.

As used herein, the term "replacement region" refers to a portion of a activation construct which becomes integrated into an endogenous chromosomal location following homologous recombination between a recombination region and a genomic sequence.

The heterologous regulatory sequences, e.g., which are provided in the replacement region, can include one or more of a variety elements, including: promoters (such as constitutive or inducible promoters), enhancers, negative regualtory elements, locus control regions, transcription factor binding sites, or combinations thereof. Promoters/enhancers which may be used to control the expression of the targeted gene *in vivo* include, but are not limited to, the cytomegalovirus (CMV) promoter/enhancer (Karasuyama et al., 1989, *J. Exp.*

Med., 169:13), the human β-actin promoter (Gunning et al. (1987) *PNAS* 84:4831-4835), the glucocorticoid-inducible promoter present in the mouse mammary tumor virus long terminal repeat (MMTV LTR) (Klessig et al. (1984) *Mol. Cell Biol.* 4:1354-1362), the long terminal repeat sequences of Moloney murine leukemia virus (MuLV LTR) (Weiss et al. (1985) *RNA Tumor Viruses*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York), the SV40 early or late region promoter (Bernoist et al. (1981) *Nature* 290:304-310; Templeton et al. (1984) *Mol. Cell Biol.*, 4:817; and Sprague et al. (1983) *J. Virol.*, 45:773), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (RSV) (Yamamoto et al., 1980, *Cell*, 22:787-797), the herpes simplex virus (HSV) thymidine kinase promoter/enhancer (Wagner et al. (1981) *PNAS* 82:3567-71), and the herpes simplex virus LAT promoter (Wolfe et al. (1992) *Nature Genetics*, 1:379-384).

In an exemplary embodiment, portions of the 5' flanking region of the human Shh gene are amplified using primers which add restriction sites, to generate the following fragments

5'-gcgcgcttcgaaGCGAGGCAGCCAGCGAGGGAGAGAGCGAGCGGGCGAGCCGGAGC-GAGGAAatcgatgcgcgc (primer 1)

5'-gcgcgcagatctGGGAAAGCGCAAGAGAGAGCGCACACGCACACACCCGCCGCGCG-CACTCGggatccgcgcgc (primer 2)

As illustrated, primer 1 includes a 5' non-coding region of the human Shh gene and is flanked by an AsuII and ClaI restriction sites. Primer 2 includes a portion of the 5' non-coding region immediately 3' to that present in primer 1. The hedgehog gene sequence is flanked by XhoII and BamHI restriction sites. The purified amplimers are cut with each of the enzymes as appropriate.

The vector pCDNA1.1 (Invitrogen) includes a CMV promoter. The plasmid is cut with with AsuII, which cleaves just 3' to the CMV promoter sequence. The AsuII/ClaI fragment of primer 1 is ligated to the AsuII cleavage site of the pcDNA vector. The ClaI/AsuII ligation destroys the AsuII site at the 3' end of a properly inserted primer 1.

The vector is then cut with BamHI, and an XhoII/BamHI fragment of primer 2 is ligated to the BamHI cleavage site. As above, the BamHI/XhoII ligation destroys the BamHI site at the 5' end of a properly inserted primer 2.

Individual colonies are selected, cut with AsuII and BamHI, and the size of the AsuII/BamHI fragment determined. Colonies in which both the primer 1 and primer 2 sequences are correctly inserted are further amplified, an cut with AsuII and BamHI to produce the gene activation construct

cgaagcgaggcagccagcgagggagagagcgagcgggcgagccggagcgaggaaATCGAAGGT
TCGAATCCTTCCCCCACCACCATCACTTTCAAAAGTCCGAAAGAATCTGCTCCCTGCTTGTGT
GTTGGAGGTCGCTGAGTAGTGCGCGAGTAAAATTTAAGCTACAACAAGGCAAGGCTTGACCGA
CAATTGCATGAAGAATCTGCTTAGGGTTAGGCGTTTTGCGCTGCTTCGCGATGTACGGGCCAG
ATATACGCGTTGACATTGATTATTGACTAGTTATTAATAGTAATCAATTACGGGGTCATTAGT
TCATAGCCCATATATGGAGTTCCGCGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGACC
GCCCAACGACCCCCGCCCATTGACGTCAATAATGACGTATGTTCCCATAGTAACGCCAATAGG
GACTTTCCATTGACGTCAATGGGTGGACTATTTACGGTAAACTGCCCACTTGGCAGTACATCA
AGTGTATCATATGCCAAGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGCA
TTATGCCCAGTACATGACCTTATGGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTCAT
CGCTATTACCATGGTGATGCGGTTTTGGCAGTACATCAATGGGCGTGGATAGCGGTTTGACTC
ACGGGGATTTCCAAGTCTCCACCCCATTGACGTCAATGGGAGTTTGTTTTGGCACCAAAATCA
ACGGGACTTTCCAAAATGTCGTAACAACTCCGCCCCATTGACGCAAATGGGCGGTAGGCGTGT
ACGGTGGGAGGTCTATATAAGCAGAGCTCTCTGGCTAACTAGAGAACCCACTGCTTACTGGCT
TATCGAAATTAATACGACTCACTATAGGGAGACCCAAGCTTGGTACCGAGCTCGGATCgatct
gggaaagcgcaagagagagcgcacacgcacacacccgccgcgcgcactcgg

In this construct, the flanking primer 1 and primer 2 sequences provide the recombination region which permits the insertion of the CMV promoter in front of the coding sequence for the human *Shh* gene. Other heterologous promoters (or other transcriptional regulatory sequences) can be inserted in a genomic *hedgehog* gene by a similar method.

In still other embodiments, the replacement region merely deletes a negative transcriptional control element of the native gene, e.g., to activate expression, or ablates a positive control element, e.g., to inhibit expression of the targeted gene.

V. Exemplary ptc therapeutic compounds.

In another embodiment, the subject method is carried out using a ptc therapeutic composition. Such compositions can be generated with, for example, compounds which bind to patched and alter its signal transduction activity, compounds which alter the binding and/or enzymatic activity of a protein (e.g., intracellular) involved in patched signal pathway, and compounds which alter the level of expression of a hedgehog protein, a patched protein or a protein involved in the intracellular signal transduction pathway of patched.

The availability of purified and recombinant *hedgehog* polypeptides facilitates the generation of assay systems which can be used to screen for drugs, such as small organic molecules, which are either agonists or antagonists of the normal cellular function of a *hedgehog* and/or patched protein, particularly their role in the pathogenesis of epithelial cell proliferation and/or differentiation. In one embodiment, the assay evaluates the ability of a compound to modulate binding between a *hedgehog* polypeptide and a *hedgehog* receptor such as *patched*. In other embodiments, the assay merely scores for the ability of a test compound to alter the signal transduction acitity of the *patched* protein. In this manner, a variety of *hedgehog* and/or *ptc* therapeutics, both proliferative and anti-proliferative in activity, can be identified. A variety of assay formats will suffice and, in light of the present disclosure, will be comprehended by skilled artisan.

In many drug screening programs which test libraries of compounds and natural extracts, high throughput assays are desirable in order to maximize the number of compounds surveyed in a given period of time. Assays which are performed in cell-free systems, such as may be derived with purified or semi-purified proteins, are often preferred as "primary" screens in that they can be generated to permit rapid development and relatively easy detection of an alteration in a molecular target which is mediated by a test compound. Moreover, the effects of cellular toxicity and/or bioavailability of the test compound can be generally ignored in the *in vitro* system, the assay instead being focused primarily on the effect of the drug on the molecular target as may be manifest in an alteration of binding affinity with receptor proteins.

Acordingly, in an exemplary screening assay for *ptc* therapeutics, the compound of interest is contacted with a mixture including a *hedgehog* receptor protein (e.g., a cell

expressing the *patched* receptor) and a hedgehog protein under conditions in which it is ordinarily capable of binding the *hedgehog* protein. To the mixture is then added a composition containing a test compound. Detection and quantification of receptor/*hedgehog* complexes provides a means for determining the test compound's efficacy at inhibiting (or potentiating) complex formation between the receptor protein and the *hedgehog* polypeptide. The efficacy of the compound can be assessed by generating dose response curves from data obtained using various concentrations of the test compound. Moreover, a control assay can also be performed to provide a baseline for comparison. In the control assay, isolated and purified *hedgehog* polypeptide is added to the receptor protein, and the formation of receptor/*hedgehog* complex is quantitated in the absence of the test compound.

In other embodiments, a ptc therapeutic of the present invention is one which disrupts the association of *patched* with *smoothened.*

Agonist and antagonists of epithelial cell growth can be distinguished, and the efficacy of the compound can be assessed, by subsequent testing with epithelial cells, e.g., in culture.

In an illustrative embodiment, the polypeptide utilized as a *hedgehog* receptor can be generated from the *patched* protein. Accordingly, an exemplary screening assay includes all or a suitable portion of the *patched* protein which can be obtained from, for example, the human *patched* gene (GenBank U43148) or other vertebrate sources (see GenBank Accession numbers U40074 for chicken *patched* and U46155 for mouse *patched*), as well as from drosophila (GenBank Accession number M28999) or other invertebrate sources. The *patched* protein can be provided in the screening assay as a whole protein (preferably expressed on the surface of a cell), or alternatively as a fragment of the full length protein which binds to *hedgehog* polypeptides, e.g., as one or both of the substantial extracellular domains (e.g. corresponding to residues Asn120-Ser438 and/or Arg770-Trp1027 of the human *patched* protein - which are also potential antagonists of *hedgehog*-dependent signal transduction). For instance, the *patched* protein can be provided in soluble form, as for example a preparation of one of the extracellular domains, or a preparation of both of the extracellular domains which are covalently connected by an unstructured linker (see, for example, Huston et al. (1988) PNAS 85:4879; and U.S. Patent No. 5,091,513). In other embodiments, the protein can be provided as part of a liposomal preparation or expressed on the surface of a cell. The *patched*

protein can derived from a recombinant gene, e.g., being ectopically expressed in a heterologous cell. For instance, the protein can be expressed on oocytes, mammalian cells (e.g., COS, CHO, 3T3 or the like), or yeast cell by standard recombinant DNA techniques. These recombinant cells can be used for receptor binding, signal transduction or gene expression assays. Marigo et al. (1996) *Development* 122:1225-1233 illustrates a binding assay of human *hedgehog* to chick *patched* protein ectopically expressed in *Xenopus laevis* oocytes. The assay system of Marigo et al. can be adapted to the present drug screening assays. As illustrated in that reference, *Shh* binds to the *patched* protein in a selective, saturable, dose-dependent manner, thus demonstrating that *patched* is a receptor for *Shh*.

Complex formation between the *hedgehog* polypeptide and a *hedgehog* receptor may be detected by a variety of techniques. For instance, modulation of the formation of complexes can be quantitated using, for example, detectably labelled proteins such as radiolabelled, fluorescently labelled, or enzymatically labelled *hedgehog* polypeptides, by immunoassay, or by chromatographic detection.

Typically, for cell-free assays, it will be desirable to immobilize either the *hedgehog* receptor or the *hedgehog* polypeptide to facilitate separation of receptor/*hedgehog* complexes from uncomplexed forms of one of the proteins, as well as to accommodate automation of the assay. In one embodiment, a fusion protein can be provided which adds a domain that allows the protein to be bound to a matrix. For example, glutathione-S-transferase/receptor (GST/receptor) fusion proteins can be adsorbed onto glutathione sepharose beads (Sigma Chemical, St. Louis, MO) or glutathione derivatized microtitre plates, which are then combined with the *hedgehog* polypeptide, e.g. an ^{35}S-labeled *hedgehog* polypeptide, and the test compound and incubated under conditions conducive to complex formation, e.g. at physiological conditions for salt and pH, though slightly more stringent conditions may be desired. Following incubation, the beads are washed to remove any unbound *hedgehog* polypeptide, and the matrix bead-bound radiolabel determined directly (e.g. beads placed in scintillant), or in the supernatant after the receptor/*hedgehog* complexes are dissociated. Alternatively, the complexes can be dissociated from the bead, separated by SDS-PAGE gel, and the level of *hedgehog* polypeptide found in the bead fraction quantitated from the gel using standard electrophoretic techniques.

Other techniques for immobilizing proteins on matrices are also available for use in the subject assay. For instance, soluble portions of the *hedgehog* receptor protein can be immobilized utilizing conjugation of biotin and streptavidin. For instance, biotinylated receptor molecules can be prepared from biotin-NHS (N-hydroxy-succinimide) using techniques well known in the art (e.g., biotinylation kit, Pierce Chemicals, Rockford, IL), and immobilized in the wells of streptavidin-coated 96 well plates (Pierce Chemical). Alternatively, antibodies reactive with the *hedgehog* receptor but which do not interfere with *hedgehog* binding can be derivatized to the wells of the plate, and the receptor trapped in the wells by antibody conjugation. As above, preparations of a *hedgehog* polypeptide and a test compound are incubated in the receptor-presenting wells of the plate, and the amount of receptor/*hedgehog* complex trapped in the well can be quantitated. Exemplary methods for detecting such complexes, in addition to those described above for the GST-immobilized complexes, include immunodetection of complexes using antibodies reactive with the *hedgehog* polypeptide, or which are reactive with the receptor protein and compete for binding with the *hedgehog* polypeptide; as well as enzyme-linked assays which rely on detecting an enzymatic activity associated with the *hedgehog* polypeptide. In the instance of the latter, the enzyme can be chemically conjugated or provided as a fusion protein with the *hedgehog* polypeptide. To illustrate, the *hedgehog* polypeptide can be chemically cross-linked or genetically fused with alkaline phosphatase, and the amount of *hedgehog* polypeptide trapped in the complex can be assessed with a chromogenic substrate of the enzyme, e.g. paranitrophenylphosphate. Likewise, a fusion protein comprising the *hedgehog* polypeptide and glutathione-S-transferase can be provided, and complex formation quantitated by detecting the GST activity using 1-chloro-2,4-dinitrobenzene (Habig et al (1974) *J Biol Chem* 249:7130).

For processes which rely on immunodetection for quantitating one of the proteins trapped in the complex, antibodies against the protein, such as the anti-*hedgehog* antibodies described herein, can be used. Alternatively, the protein to be detected in the complex can be "epitope tagged" in the form of a fusion protein which includes, in addition to the *hedgehog* polypeptide or *hedgehog* receptor sequence, a second polypeptide for which antibodies are readily available (e.g. from commercial sources). For instance, the GST fusion proteins described above can also be used for quantification of binding using antibodies against the

GST moiety. Other useful epitope tags include myc-epitopes (e.g., see Ellison et al. (1991) *J Biol Chem* 266:21150-21157) which includes a 10-residue sequence from c-myc, as well as the pFLAG system (International Biotechnologies, Inc.) or the pEZZ-protein A system (Pharamacia, NJ).

Where the desired portion of the *hedgehog* receptor (or other *hedgehog* binding molecule) cannot be provided in soluble form, liposomal vesicles can be used to provide manipulatable and isolatable sources of the receptor. For example, both authentic and recombinant forms of the *patched* protein can be reconstituted in artificial lipid vesicles (e.g. phosphatidylcholine liposomes) or in cell membrane-derived vesicles (see, for example, Bear et al. (1992) *Cell* 68:809-818; Newton et al. (1983) *Biochemistry* 22:6110-6117; and Reber et al. (1987) *J Biol Chem* 262:11369-11374).

In addition to cell-free assays, such as described above, the readily available source of *hedgehog* proteins provided by the art also facilitates the generation of cell-based assays for identifying small molecule agonists/antagonists and the like. Analogous to the cell-based assays described above for screening combinatorial libraries, cells which are sensitive to *hedgehog* induction, e.g. *patched*-expressing cells or other epithelially-derived cells sensitive to *hedgehog* induction, can be contacted with a *hedgehog* protein and a test agent of interest, with the assay scoring for anything from simple binding to the cell to modulation in *hedgehog* inductive responses by the target cell in the presence and absence of the test agent. As with the cell-free assays, agents which produce a statistically significant change in *hedgehog* activities (either inhibition or potentiation) can be identified.

In other emdodiments, the cell-based assay scores for agents which disrupt association of patched and *smoothened* proteins, e.g., in the cell surface membrane or liposomal preparation.

In addition to characterizing cells that naturally express the *patched* protein, cells which have been genetically engineered to ectopically express *patched* can be utilized for drug screening assays. As an example, cells which either express low levels or lack expression of the *patched* protein, e.g. *Xenopus laevis* oocytes, COS cells or yeast cells, can be genetically modified using standard techniques to ectopically express the *patched* protein. (see Marigo et al., *supra*).

The resulting recombinant cells, e.g., which express a functional *patched* receptor, can be utilized in receptor binding assays to identify agonist or anatagonsts of *hedgehog* binding. Binding assays can be performed using whole cells. Furthermore, the recombinant cells of the present invention can be engineered to include other heterolgous genes encoding proteins involved in *hedgehog*-dependent siganl pathways. For example, the gene products of one or more of *smoothened, costal-2* and/or *fused* can be co-expressed with *patched* in the reagent cell, with assays being sensitive to the functional reconstituion of the *hedgehog* signal transduction cascade.

Alternatively, liposomal preparations using reconstituted *patched* protein can be utilized. *Patched* protein purified from detergent extracts from both authentic and recombinant origins can be reconstituted in in artificial lipid vesicles (e.g. phosphatidylcholine liposomes) or in cell membrane-derived vesicles (see, for example, Bear et al. (1992) *Cell* 68:809-818; Newton et al. (1983) *Biochemistry* 22:6110-6117; and Reber et al. (1987) *J Biol Chem* 262:11369-11374). The lamellar structure and size of the resulting liposomes can be characterized using electron microscopy. External orientation of the *patched* protein in the reconstituted membranes can be demonstrated, for example, by immunoelectron microscopy. The *hedgehog* protein binding activity of liposomes containing *patched* and liposomes without the protein in the presence of candidate agents can be compared in order to identify potential modulators of the *hedgehog-patched* interaction.

The *hedgehog* protein used in these cell-based assays can be provided as a purified source (natural or recombinant in origin), or in the form of cells/tissue which express the protein and which are co-cultured with the target cells. As in the cell-free assays, where simple binding (rather than induction) is the hedgehog activity scored for in the assay, the protein can be labelled by any of the above-mentioned techniques, e.g., fluorescently, enzymatically or radioactively, or detected by immunoassay.

In addition to binding studies, functional assays can be used to identified modulators, i.e., agonists or antagonists, of *hedgehog* or *patched* activities. By detecting changes in intracellular signals, such as alterations in second messengers or gene expression, in *patched*-expressing cells contacted with a test agent, candidate agonists and antagonists to *patched* signaling can be identified.

A number of gene products have been implicated in *patched*-mediated signal transduction, including *patched*, the transcription factor *cubitus interruptus* (ci), the serine/threonine kinase *fused* (fu) and the gene products of *costal-2*, *smoothened* and *suppressor of fused*.

The interaction of a hedgehog protein with *patched* sets in motion a cascade involving the activation and inhibition of downstream effectors, the ultimate consequence of which is, in some instances, a detectable change in the transcription or translation of a gene. Potential transcriptional targets of *patched* signaling are the *patched* gene itself (Hidalgo and Ingham, 1990 *Development* 110, 291-301; Marigo et al., 1996) and the vertebrate homologs of the drosophila cubitus interruptus gene, the *GLI* genes (Hui et al. (1994) *Dev Biol* 162:402-413). *Patched* gene expression has been shown to be induced in cells of the limb bud and the neural plate that are responsive to *Shh*. (Marigo et al. (1996) *PNAS*, in press; Marigo et al. (1996) *Development* 122:1225-1233). The *GLI* genes encode putative transcription factors having zinc finger DNA binding domains (Orenic et al. (1990) *Genes & Dev* 4:1053-1067; Kinzler et al. (1990) *Mol Cell Biol* 10:634-642). Transcription of the *GLI* gene has been reported to be upregulated in response to *hedgehog* in limb buds, while transcription of the *GLI3* gene is downregulated in response to *hedgehog* induction (Marigo et al. (1996) *Development* 122:1225-1233). By selecting transcriptional regulatory sequences from such target genes, e.g. from *patched* or *GLI* genes, that are responsible for the up- or down regulation of these genes in response to *patched* signalling, and operatively linking such promoters to a reporter gene, one can derive a transcription based assay which is sensitive to the ability of a specific test compound to modify *patched* signalling pathways. Expression of the reporter gene, thus, provides a valuable screening tool for the development of compounds that act as agonists or antagonists of *ptc* induction of differentiation/quiescence.

Reporter gene based assays of this invention measure the end stage of the above described cascade of events, e.g., transcriptional modulation. Accordingly, in practicing one embodiment of the assay, a reporter gene construct is inserted into the reagent cell in order to generate a detection signal dependent on *ptc* signaling. To identify potential regulatory elements responsive to *ptc* signaling present in the transcriptional regulatory sequence of a target gene, nested deletions of genomic clones of the target gene can be constructed using

standard techniques. See, for example, Current Protocols in Molecular Biology, Ausubel, F.M. et al. (eds.) Greene Publishing Associates, (1989); U.S. Patent 5,266,488; Sato et al. (1995) *J Biol Chem* 270:10314-10322; and Kube et al. (1995) *Cytokine* 7:1-7. A nested set of DNA fragments from the gene's 5'-flanking region are placed upstream of a reporter gene, such as the luciferase gene, and assayed for their ability to direct reporter gene expression in *patched* expressing cells. Host cells transiently transfected with reporter gene constructs can be scored for the induction of expression of the reporter gene in the presence and absence of *hedgehog* to determine regulatory sequences which are responsice to *patched*-dependent signalling.

In practicing one embodiment of the assay, a reporter gene construct is inserted into the reagent cell in order to generate a detection signal dependent on second messengers generated by induction with *hedgehog* protein. Typically, the reporter gene construct will include a reporter gene in operative linkage with one or more transcriptional regulatory elements responsive to the *hedgehog* activity, with the level of expression of the reporter gene providing the *hedgehog*-dependent detection signal. The amount of transcription from the reporter gene may be measured using any method known to those of skill in the art to be suitable. For example, mRNA expression from the reporter gene may be detected using RNAse protection or RNA-based PCR, or the protein product of the reporter gene may be identified by a characteristic stain or an intrinsic activity. The amount of expression from the reporter gene is then compared to the amount of expression in either the same cell in the absence of the test compound (or *hedgehog*) or it may be compared with the amount of transcription in a substantially identical cell that lacks the target receptor protein. Any statistically or otherwise significant difference in the amount of transcription indicates that the test compound has in some manner altered the signal transduction of the *patched* protein, e.g., the test compound is a potential ptc therapeutic.

As described in further detail below, in preferred embodiments the gene product of the reporter is detected by an intrinsic activity associated with that product. For instance, the reporter gene may encode a gene product that, by enzymatic activity, gives rise to a detection signal based on color, fluorescence, or luminescence. In other preferred embodiments, the reporter or marker gene provides a selective growth advantage, e.g., the reporter gene may

enhance cell viability, relieve a cell nutritional requirement, and/or provide resistance to a drug.

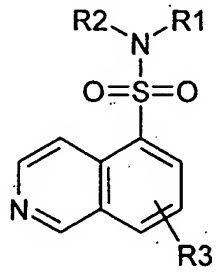
Preferred reporter genes are those that are readily detectable. The reporter gene may also be included in the construct in the form of a fusion gene with a gene that includes desired transcriptional regulatory sequences or exhibits other desirable properties. Examples of reporter genes include, but are not limited to CAT (chloramphenicol acetyl transferase) (Alton and Vapnek (1979), Nature 282: 864-869) luciferase, and other enzyme detection systems, such as beta-galactosidase; firefly luciferase (deWet et al. (1987), Mol. Cell. Biol. 7:725-737); bacterial luciferase (Engebrecht and Silverman (1984), PNAS 1: 4154-4158; Baldwin et al. (1984), Biochemistry 23: 3663-3667); alkaline phosphatase (Toh et al. (1989) Eur. J. Biochem. 182: 231-238, Hall et al. (1983) J. Mol. Appl. Gen. 2: 101), human placental secreted alkaline phosphatase (Cullen and Malim (1992) Methods in Enzymol. 216:362-368).

Transcriptional control elements which may be included in a reporter gene construct include, but are not limited to, promoters, enhancers, and repressor and activator binding sites. Suitable transcriptional regulatory elements may be derived from the transcriptional regulatory regions of genes whose expression is induced after modulation of a *patched* signal transduction pathway. The characteristics of preferred genes from which the transcriptional control elements are derived include, but are not limited to, low or undetectable expression in quiescent cells, rapid induction at the transcriptional level within minutes of extracellular simulation, induction that is transient and independent of new protein synthesis, subsequent shut-off of transcription requires new protein synthesis, and mRNAs transcribed from these genes have a short half-life. It is not necessary for all of these properties to be present.

In yet other embodiments, second messenger generation can be measured directly in the detection step, such as mobilization of intracellular calcium, phospholipid metabolism or adenylate cyclase activity are quantitated, for instance, the products of phospholipid hydrolysis IP_3, DAG or cAMP could be measured For example, recent studies have implicated protein kinase A (PKA) as a possible component of *hedgehog/patched* signaling (Hammerschmidt et al. (1996) *Genes & Dev* 10:647). High PKA activity has been shown to antagonize *hedgehog* signaling in these systems. Although it is unclear whether PKA acts directly downstream or in parallel with *hedgehog* signaling, it is possible that *hedgehog* signalling occurs via inhibition

of PKA activity. Thus, detection of PKA activity provides a potential readout for the instant assays.

In a preferred embodiment, the *ptc* therapeutic is a PKA inhibitor. A variety of PKA inhibitors are known in the art, including both peptidyl and organic compounds. For instance, the *ptc* therapeutic can be a 5-isoquinolinesulfonamide, such as represented in the general formula:



wherein,

R_1 and R_2 each can independently represent hydrogen, and as valence and stability permit a lower alkyl, a lower alkenyl, a lower alkynyl, a carbonyl (such as a carboxyl, an ester, a formate, or a ketone), a thiocarbonyl (such as a thioester, a thioacetate, or a thioformate), an amino, an acylamino, an amido, a cyano, a nitro, an azido, a sulfate, a sulfonate, a sulfonamido, $-(CH_2)_m-R_8$, $-(CH_2)_m-OH$, $-(CH_2)_m-O$-lower alkyl, $-(CH_2)_m-O$-lower alkenyl, $-(CH_2)_n-O-(CH_2)_m-R_8$, $-(CH_2)_m-SH$, $-(CH_2)_m-S$-lower alkyl, $-(CH_2)_m-S$-lower alkenyl, $-(CH_2)_n-S-(CH_2)_m-R_8$, or

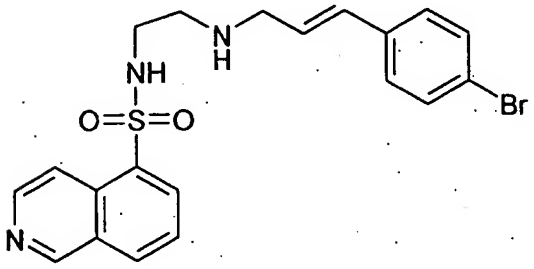
R_1 and R_2 taken together with N form a heterocycle (substituted or unsubstituted);

R_3 is absent or represents one or more substitutions to the isoquinoline ring such as a lower alkyl, a lower alkenyl, a lower alkynyl, a carbonyl (such as a carboxyl, an ester, a formate, or a ketone), a thiocarbonyl (such as a thioester, a thioacetate, or a thioformate), an amino, an acylamino, an amido, a cyano, a nitro, an azido, a sulfate, a sulfonate, a sulfonamido, $-(CH_2)_m-R_8$, $-(CH_2)_m-OH$, $-(CH_2)_m-O$-lower alkyl, $-(CH_2)_m-O$-lower alkenyl, $-(CH_2)_n-O-(CH_2)_m-R_8$, $-(CH_2)_m-SH$, $-(CH_2)_m-S$-lower alkyl, $-(CH_2)_m-S$-lower alkenyl, $-(CH_2)_n-S-(CH_2)_m-R_8$;

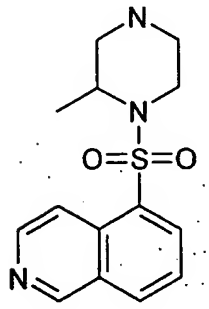
R_8 represents a substituted or unsubstituted aryl, aralkyl, cycloalkyl, cycloalkenyl, or heterocycle; and

n and m are independently for each occurrence zero or an integer in the range of 1 to 6.

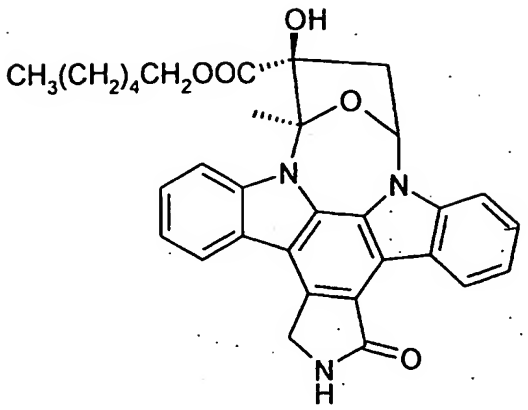
In a preferred embodiment, the PKA inhibitor is N-[2-((p-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide (H-89; Calbiochem Cat. No. 371963), e.g., having the formula:



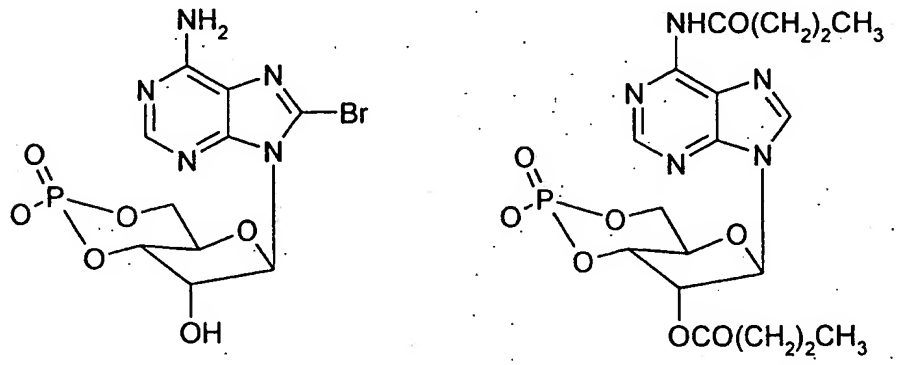
In another embodiment, the PKA inhibitor is 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7; Calbiochem Cat. No. 371955), e.g., having the formula:



In still other embodiments, the PKA inhibitor is KT5720 (Calbiochem Cat. No. 420315), having the structure



A variety of nucleoside analogs are also useful as PKA inhibitors. For example, the subject method can be carried out cyclic AMP analogs which inhibit the kinase activity of PKA, as for example, 8-bromo-cAMP or dibutyryl-cAMP



Exemplary peptidyl inhibitors of PKA activity include the PKA Heat Stable Inhibitor (isoform α; see, for example, Calbiochem Cat. No. 539488, and Wen et al. (1995) *J Biol Chem* 270:2041).

Certain *hedehog* receptors may stimulate the activity of phospholipases. Inositol lipids can be extracted and analyzed using standard lipid extraction techniques. Water soluble derivatives of all three inositol lipids (IP_1, IP_2, IP_3) can also be quantitated using radiolabelling techniques or HPLC.

The mobilization of intracellular calcium or the influx of calcium from outside the cell may be a response to *hedgehog* stimulation or lack there of. Calcium flux in the reagent cell can be measured using standard techniques. The choice of the appropriate calcium indicator, fluorescent, bioluminescent, metallochromic, or Ca^{++}-sensitive microelectrodes depends on

the cell type and the magnitude and time constant of the event under study (Borle (1990) *Environ Health Perspect* 84:45-56). As an exemplary method of Ca^{++} detection, cells could be loaded with the Ca^{++}sensitive fluorescent dye fura-2 or indo-1, using standard methods, and any change in Ca^{++} measured using a fluorometer.

In certain embodiments of the assay, it may be desirable to screen for changes in cellular phosphorylation. As an example, the drosophila gene *fused* (fu) which encodes a serine/threonine kinase has been identified as a potential downstream target in *hedgehog* signaling. (Preat et al., 1990 *Nature* 347, 87-89; Therond et al. 1993, *Mech. Dev.* 44. 65-80). The ability of compounds to modulate serine/threonine kinase activation could be screened using colony immunoblotting (Lyons and Nelson (1984) *Proc. Natl. Acad. Sci. USA* 81:7426-7430) using antibodies against phosphorylated serine or threonine residues. Reagents for performing such assays are commercially available, for example, phosphoserine and phosphothreonine specific antibodies which measure increases in phosphorylation of those residues can be purchased from comercial sources.

In yet another embodiment, the *ptc* therapeutic is an antisense molecule which inhibits expression of a protein involved in a *patched*-mediated signal transduction pathway. To illustrate, by inhibiting the expression of a protein which are involved in *patched* signals, such as fused, costal-2, smoothened and/or Gli genes, the ability of the patched signal pathway(s) to inhibit proliferation of a cell can be altered, e.g., potentiated or repressed.

As used herein, "antisense" therapy refers to administration or *in situ* generation of oligonucleotide probes or their derivatives which specifically hybridize (e.g. bind) under cellular conditions with cellular mRNA and/or genomic DNA encoding a *hedgehog* protein, patched, or a protein involved in patched-mediated signal transduction. The hybridization should inhibit expression of that protein, e.g. by inhibiting transcription and/or translation. The binding may be by conventional base pair complementarity, or, for example, in the case of binding to DNA duplexes, through specific interactions in the major groove of the double helix. In general, "antisense" therapy refers to the range of techniques generally employed in the art, and includes any therapy which relies on specific binding to oligonucleotide sequences.

An antisense construct of the present invention can be delivered, for example, as an expression plasmid which, when transcribed in the cell, produces RNA which is complementary to at least a unique portion of the target cellular mRNA. Alternatively, the antisense construct is an oligonucleotide probe which is generated *ex vivo* and which, when introduced into the cell causes inhibition of expression by hybridizing with the mRNA and/or genomic sequences of a target gene. Such oligonucleotide probes are preferably modified oligonucleotide which are resistant to endogenous nucleases, e.g. exonucleases and/or endonucleases, and is therefore stable *in vivo*. Exemplary nucleic acid molecules for use as antisense oligonucleotides are phosphoramidate, phosphothioate and methylphosphonate analogs of DNA (see also U.S. Patents 5,176,996; 5,264,564; and 5,256,775). Additionally, general approaches to constructing oligomers useful in antisense therapy have been reviewed, for example, by Van der Krol et al. (1988) *Biotechniques* 6:958-976; and Stein et al. (1988) *Cancer Res* 48:2659-2668.

Several considerations should be taken into account when constructing antisense oligonucleotides for the use in the methods of the invention: (1) oligos should have a GC content of 50% or more; (2) avoid sequences with stretches of 3 or more G's; and (3) oligonucleotides should not be longer than 25-26 mers. When testing an antisense oligonucleotide, a mismatched control can be constructed. The controls can be generated by reversing the sequence order of the corresponding antisense oligonucleotide in order to conserve the same ratio of bases.

In an illustrative embodiment, the *ptc* therapeutic can be an antisense construct for inhibiting the expression of *patched*, e.g., to mimic the inhibition of *patched* by *hedgehog*. Exemplary antisense constructs include:

5'-GTCCTGGCGCCGCCGCCGCCGTCGCC

5'-TTCCGATGACCGGCCTTTCGCGGTGA

5'-GTGCACGGAAAGGTGCAGGCCACACT

VI. Exemplary pharmaceutical preparations of hedgehog and ptc therapeutics

The source of the hedgehog and ptc therapeutics to be formulated will depend on the particular form of the agent. Small organic molecules and peptidyl fragments can be chemically synthesized and provided in a pure form suitable for pharmaceutical/cosmetic usage. Products of natural extracts can be purified according to techniques known in the art. For example, the Cox et al. U.S. Patent 5,286,654 describes a method for purifying naturally occurring forms of a secreted protein and can be adapted for purification of hedgehog polypeptides. Recombinant sources of hedgehog polypeptides are also available. For example, the gene encoding *hedgehog* polypeptides, are known, *inter alia*, from PCT publications WO 95/18856 and WO 96/17924.

Those of skill in treating epithelial tissues can determine the effective amount of an hedgehog or ptc therapeutic to be formulated in a pharmaceutical or cosmetic preparation.

The hedgehog or ptc therapeutic formulations used in the method of the invention are most preferably applied in the form of appropriate compositions. As appropriate compositions there may be cited all compositions usually employed for systemically or topically administering drugs. The pharmaceutically acceptable carrier should be substantially inert, so as not to act with the active component. Suitable inert carriers include water, alcohol polyethylene glycol, mineral oil or petroleum gel, propylene glycol and the like.

To prepare the pharmaceutical compositions of this invention, an effective amount of the particular hedgehog or ptc therapeutic as the active ingredient is combined in intimate admixture with a pharmaceutically acceptable carrier, which carrier may take a wide variety of forms depending on the form of preparation desired for administration. These pharmaceutical compositions are desirable in unitary dosage form suitable, particularly, for administration orally, rectally, percutaneously, or by parenteral injection. For example, in preparing the compositions in oral dosage form, any of the usual pharmaceutical media may be employed such as, for example, water, glycols, oils, alcohols and the like in the case of oral liquid preparations such as suspensions, syrups, elixirs and solutions; or solid carriers such as starches, sugars, kaolin, lubricants, binders, disintegrating agents and the like in the case of powders, pills, capsules, and tablets. Because of their ease in administration, tablets and capsules represents the most advantageous oral dosage unit form, in which case solid

pharmaceutical carriers are obviously employed. For parenteral compositions, the carrier will usually comprise sterile water, at least in large part, though other ingredients, for example, to aid solubility, may be included. Injectable solutions, for example, may be prepared in which the carrier comprises saline solution, glucose solution or a mixture of saline and glucose solution. Injectable suspensions may also be prepared in which case appropriate liquid carriers, suspending agents and the like may be employed. Also included are solid form preparations which are intended to be converted, shortly before use, to liquid form preparations. In the compositons suitable for percutaneous administration, the carrier optionally comprises a penetration enhancing agent and/or a suitable wetting agent, optionally combined with suitable additives of any nature in minor proportions, which additives do not introduce a significant deleterious effect on the skin.

In addition to the direct topical application of the preparations they can be topically administered by other methods, for example, encapsulated in a temperature and/or pressure sensitive matrix or in film or solid carrier which is soluble in body fluids and the like for subsequent release, preferably sustained-release of the active component.

As appropriate compositions for topical application there may be cited all compositions usually employed for topically administering therapeuitcs, e.g., creams, gellies, dressings, shampoos, tinctures, pastes, ointments, salves, powders, liquid or semiliquid formulation and the like. Application of said compositions may be by aerosol e.g. with a propellent such as nitrogen carbon dioxide, a freon, or without a propellent such as a pump spray, drops, lotions, or a semisolid such as a thickened composition which can be applied by a swab. In particular compositions, semisolid compositions such as salves, creams, pastes, gellies, ointments and the like will conveniently be used.

It is especially advantageous to formulate the subject compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used in the specification and claims herein refers to physically discreate units suitable as unitary dosages, each unit containing a predetermined quantity of active ingredient calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. Examples of such dosage unit forms are tablets (including scored or coated tablets), capsules, pills, powders

packets, wafers, injectable solutions or suspensions, teaspoonfuls, tablespoonfuls and the like, and segregated multiples thereof.

The pharmaceutical preparations of the present invention can be used, as stated above, for the many applications whcih can be considered cosmetic uses. Cosmetic compositions known in the art, preferably hypoallergic and pH controlled are especially preferred, and include toilet waters, packs, lotions, skin milks or milky lotions. The preparations contain, besides the hedgehog or ptc therapeutic, components usually employed in such preparations. Examples of such components are oils, fats, waxes, surfactants, humectants, thickening agents, antioxidants, viscosity stabilizers, chelating agents, buffers, preservatives, perfumes, dyestuffs, lower alkanols, and the like. If desired, further ingredients may be incorporated in the compositions, e.g. antiinflammatory agents, antibacterials, antifungals, disinfectants, vitamins, sunscreens, antibiotics, or other anti-acne agents.

Examples of oils comprise fats and oils such as olive oil and hydrogenated oils; waxes such as beeswax and lanolin; hydrocarbons such as liquid paraffin, ceresin, and squalane; fatty acids such as stearic acid and oleic acid; alcohols such as cetyl alcohol, stearyl alcohol, lanolin alcohol, and hexadecanol; and esters such as isopropyl myristate, isopropyl palmitate and butyl stearate. As examples of surfactants there may be cited anionic surfactants such as sodium stearate, sodium cetylsulfate, polyoxyethylene laurylether phosphate, sodium N-acyl glutamate; cationic surfactants such as stearyldimethylbenzylammonium chloride and stearyltrimethylammonium chloride; ampholytic surfactants such as alkylaminoethylglycine hydrocloride solutions and lecithin; and nonionic surfactants such as glycerin monostearate, sorbitan monostearate, sucrose fatty acid esters, propylene glycol monostearate, polyoxyethylene oleylether, polyethylene glycol monostearate, polyoxyethylene sorbitan monopalmitate, polyoxyethylene coconut fatty acid monoethanolamide, polyoxypropylene glycol (e.g. the materials sold under the trademark "Pluronic"), polyoxyethylene castor oil, and polyoxyethylene lanolin. Examples of humectants include glycerin, 1,3-butylene glycol, and propylene glycol; examples of lower alcohols include ethanol and isopropanol; examples of thickening agents include xanthan gum, hydroxypropyl cellulose, hydroxypropyl methyl cellulose, polyethylene glycol and sodium carboxymethyl cellulose; examples of antioxidants comprise butylated hydroxytoluene, butylated hydroxyanisole, propyl gallate, citric acid and

ethoxyquin; examples of chelating agents include disodium edetate and ethanehydroxy diphosphate; examples of buffers comprise citric acid, sodium citrate, boric acid, borax, and disodium hydrogen phosphate; and examples of preservatives are methyl parahydroxybenzoate, ethyl parahydroxybenzoate, dehydroacetic acid, salicylic acid and benzoic acid.

For preparing ointments, creams, toilet waters, skin milks, and the like, typically from 0.01 to 10% in particular from 0.1 to 5% and more in particular from 0.2 to 2.5% of the active ingredient, e.g., of the hedgehog or ptc therapeutic, will be incorporated in the compositions. In ointments or creams, the carrier for example consists of 1 to 20%, in particular 5 to 15% of a humectant, 0.1 to 10% in particular from 0.5 to 5% of a thickener and water; or said carrier may consist of 70 to 99%, in particular 20 to 95% of a surfactant, and 0 to 20%, in particular 2.5 to 15% of a fat; or 80 to 99.9% in particular 90 to 99% of a thickener; or 5 to 15% of a surfactant, 2-15% of a humectant, 0 to 80% of an oil, very small (< 2%) amounts of preservative, coloring agent and/or perfume, and water. In a toilet water, the carrier for example consists of 2 to 10% of a lower alcohol, 0.1 to 10% or in particular 0.5 to 1% of a surfactant, 1 to 20%, in particular 3 to 7% of a humectant, 0 to 5% of a buffer, water and small amounts (< 2%) of preservative, dyestuff and/or perfume. In a skin milk, the carrier typically consists of 10-50% of oil, 1 to 10% of surfactant, 50-80% of water and 0 to 3% of preservative and/or perfume. In the aforementioned preparations, all % symbols refer to weight by weight percentage.

Particular compositions for use in the method of the present invention are those wherein the hedgehog or ptc therapeutic is formulated in liposome-containing compositions. Liposomes are artificial vesicles formed by amphiphatic molecules such as polar lipids, for example, phosphatidyl cholines, ethanolamines and serines, sphingomyelins, cardiolipins, plasmalogens, phosphatidic acids and cerebiosides. Liposomes are formed when suitable amphiphathic molecules are allowed to swell in water or aqueous solutions to form liquid crystals usually of multilayer structure comprised of many bilayers separated from each other by aqueous material (also referred to as coarse liposomes). Another type of liposome known to be consisting of a single bilayer encapsulating aqueous material is referred to as a unilamellar

vesicle. If water-soluble materials are included in the aqueous phase during the swelling of the lipids they become entrapped in the aqueous layer between the lipid bilayers.

Water-soluble active ingredients such as, for example, various salt forms of a hedgehog polypeptide, are encapsulated in the aqueous spaces between the molecular layers. The lipid soluble active ingredient of hedgehog or ptc therapeutic, such as an organic mimetic, is predominantly incorporated into the lipid layers, although polar head groups may protude from the layer into the aqueous space. The encapsulation of these compounds can be achieved by a number of methods. The method most commonly used involves casting a thin film of phospholipid onto the walls of a flask by evaporation from an organic solvent. When this film is dispersed in a suitable aqueous medium, multilamellar liposomes are formed. Upon suitable sonication, the coarse liposomes form smaller similarly closed vesicles.

Water-soluble active ingredients are usually incorporated by dispersing the cast film with an aqueous solution of the compound. The unencapsulated compound is then removed by centrifugation, chromatography, dialysis or other art-known suitable procedures. The lipid-soluble active ingredient is usually incorporated by dissolving it in the organic solvent with the phospholipid prior to casting the film. If the solubility of the material in the lipid phase is not exceeded or the amount present is not in excess of that which can be bound to the lipid, liposomes prepared by the above method usually contain most of the material bound in the lipid bilayers; separation of the liposomes from unencapsulated material is not required.

A particularly convenient method for preparing liposome formulated forms of hedgehog and ptc therapeutics is the method described in EP-A-253,619, incorporated herein by reference. In this method, single bilayered liposomes containing encapsulated active ingredients are prepared by dissolving the lipid component in an organic medium, injecting the organic solution of the lipid component under pressure into an aqueous component while simultaneously mixing the organic and aqueous components with a high speed homogenizer or mixing means, whereupon the liposomes are formed spontaneously.

The single bilayered liposomes containing the encapsulated hedgehog or ptc therapeutic can be employed directly or they can be employed in a suitable pharmaceutically acceptable carrier for topical administration. The viscosity of the liposomes can be increased by the addition of one or more suitable thickening agents such as, for example xanthan gum,

hydroxypropyl cellulose, hydroxypropyl methylcellulose and mixtures thereof. The aqueous component may consist of water alone or it may contain electrolytes, buffered systems and other ingredients, such as, for example, preservatives. Suitable electrolytes which can be employed include metal salts such as alkali metal and alkaline earth metal salts. The preferred metal salts are calcium chloride, sodium chloride and potassium chloride. The concentration of the electrolyte may vary from zero to 260 mM, preferably from 5 mM to 160 mM. The aqueous component is placed in a suitable vessel which can be adapted to effect homogenization by effecting great turbulence during the injection of the organic component. Homogenization of the two components can be accomplished within the vessel, or, alternatively, the aqueous and organic components may be injected separately into a mixing means which is located outside the vessel. In the latter case, the liposomes are formed in the mixing means and then transferred to another vessel for collection purpose.

The organic component consists of a suitable non-toxic, pharmaceutically acceptable solvent such as, for example ethanol, glycerol, propylene glycol and polyethylene glycol, and a suitable phospholipid which is soluble in the solvent. Suitable phospholipids which can be employed include lecithin, phosphatidylcholine, phosphatydylserine, phosphatidylethanol-amine, phosphatidylinositol, lysophosphatidylcholine and phospha-tidyl glycerol, for example. Other lipophilic additives may be employed in order to selectively modify the characteristics of the liposomes. Examples of such other additives include stearylamine, phosphatidic acid, tocopherol, cholesterol and lanolin extracts.

In addition, other ingredients which can prevent oxidation of the phospholipids may be added to the organic component. Examples of such other ingredients include tocopherol, butylated hydroxyanisole, butylated hydroxytoluene, ascorbyl palmitate and ascorbyl oleate. Preservatives such a benzoic acid, methyl paraben and propyl paraben may also be added.

Apart from the above-described compositions, use may be made of covers, e.g. plasters, bandages, dressings, gauze pads and the like, containing an appropriate amount of a hedgehog or ptc therapeutic. In some cases use may be made of plasters, bandages, dressings, gauze pads and the like which have been impregnated with a topical formulation containing the therapeutic formulation.

Exemplification

The invention now being generally described, it will be more readily understood by reference to the following examples which are included merely for purposes of illustration of certain aspects and embodiments of the present invention, and are not intended to limit the invention.

Purification of hedgehog protein.

Human sonic hedgehog protein (residues 24-197) was expressed in the baculovirus/insect cell system (Roelink et al. (1995) *Cell* 81:445-455). The conditioned medium was loaded onto Fast Flo SP agarose equilibrated with 50 mM potassium phosphate, 0.5 mM DTT, pH 7.0. The column was washed with this buffer, and then eluted with a gradient to 10. M NaCl. Fractions were assayed for the induction of alkaline phosphatase activity on mesenchymal stem cells (C3H10T1/2 cells, see, e.g., Wang et al. (1993) *Growth Factors* 9:57-71) and then pooled on the basis of this activity and also by purity on SDS gels. The pooled material was concentrated on an Amicon ultra filtration unit (PM10 membrane) and diafiltered against 10 mM Tris, pH 7.4, 0.5 mM DTT. Protein was estimated by the Bradford method using gamma globulin as a standard.

Preparation of collagen sponge.

Collagen sponge was washed extensively in MilliQ water to remove any surfactants and additives from the manufacturer. The sponge was then washed in 70% ethanol, then dried in vacuo.

Preparation of implants.

Protein was added to 1.0 - 1.5 mm by 8-10 mm pieces of collagen sponge (1.5-3.0 mg in weight). In some cases zinc sulfate was added to a final concentration of 0.2 mM before the

hedgehog protein was added to the collagen sponge. The reconstituted sponges were then frozen and lyophilized.

Implantation.

Sponges were implanted either subcutaneously in the thoracic region of Sprague Dawley rats (4-8 weeks old) or in the thigh muscle of rabbits (11-14 weeks old). Animals were maintained for 2-5 weeks before removing the implant. The implant was then fixed in 4% formalin or 4% paraformaldehyde and then embedded in JB-4 resin. Sections were stained with toluidine blue (Wang et al. (1988) *PNAS* 85:9484-9488).

The induction of new hair follicles, sebaceous glands, and other dermal structures were identified by its distinctive morphology. The careful subcutaneous or intramuscular placement of our implants and the careful removal of these implants preclude the possibility of contamination from existing dermal structures. Also, the appearance of more immature hair follicles is seen in the implants of shorter (2 week) duration.

Biopsy slides were obtained from an intramuscular implant taken out of a rabbit muscle at three weeks and stained with hematoxylin and eosin. Similar slides were examined of an intramuscular implant, rabbit muscle, three weeks, stained with toluidine blue. Slides of certain samples revealed a tissue morphology indicating the presence of follicle- and hair-like structures forming in the intramuscular tissue.

Hair induction by Shh

As a follow-up to the above experiments, hedgehog-loaded collagen sponges were implanted under the shaved skin of mice. As indicated in Figures 1A-C, the hedgehog preparations were able to induce hair growth over the implants. Moreover, Ihh protein modified at the C terminus with a Von Willebrand's factor collagen binding site was active in hair growth, indicating a localized inducing activity of the implanted protein.

All of the above-cited references and publications are hereby incorporated by reference.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific polypeptides, nucleic acids, methods, assays and reagents described herein. Such equivalents are considered to be within the scope of this invention.

We claim:

1. A method for modulating the growth state of an epithelial cell comprising ectopically contacting the epithelial cell with an amount of an agent effective to alter the rate of proliferation of the epithelial cell, wherein the agent is selected from the group consisting of a hedgehog therapeutic and a ptc therapeutic.

2. A method for modulating the growth state of an epithelial tissue comprising ectopically contacting the tissue with an amount of an agent effective to alter the rate of proliferation of the epithelial cells in the tissue, wherein the agent is selected from the group consisting of a hedgehog therapeutic and a ptc therapeutic.

3. A method for inducing the formation of skin, comprising treating the skin with an amount of an agent effective to induce the formation of new skin tissue, wherein the agent is selected from the group consisting of a hedgehog therapeutic and a ptc therapeutic.

4. A method for inducing growth of hair on an animal, comprising treating the animal with an amount of an agent effective to induce growth of hair, wherein the agent is selected from the group consisting of a hedgehog therapeutic and a ptc therapeutic which induce proliferation of hair follicle keratinocytes.

5. The method of claim 1 or 2, wherein the agent increases the rate of proliferation of epithelial cells.

6. The method of claim 1 or 2, wherein the agent decreases the rate of proliferation of epithelial cells.

7. The method of claim 1 or 2, wherein the epithelial cell is a cutaneous epithelial cell.

8. The method of claim 7, wherein the epithelial cell is a dermal keratinocyte.

9. The method of claim 7, wherein the epithelial cell is a mucosal epithelial cell.

10. The method of claim 7, wherein the epithelial cell is an epithelial stem cell.

11. The method of claim 7, wherein the epithelial cell is a hair follicle stem cell.

12. The method of claim 1, wherein the cell is in culture, and the agent is provided as a cell culture additive.

13. The method of claim 1, wherein the cell is treated in an animal and the agent is administered to the animal as a therapeutic composition.

14. The method of claim 1, wherein the epithelial tissue is in tissue culture, and the agent is provided as a tissue culture additive.

15. The method of claim 1, wherein the epithelial tissue is treated in an animal and the agent is administered to the animal as a therapeutic composition.

16. The method of claim 3, 4, 13 or 15, wherein the agent is applied topically.

17. The method of claim 1, 2, 3 or 4, wherein the agent is a hedgehog therapeutic.

18. The method of claim 17, wherein the hedgehog therapeutic is a polypeptide including a *hedgehog* polypeptide sequence of at least a bioactive extracellular portion of a hedgehog protein.

19. The method of claim 18, wherein the polypeptide includes at least 50 amino acids residues of an N-terminal half of the *hedgehog* protein.

20. The method of claim 18, wherein the polypeptide includes at least 100 amino acids of an extracellular domain of the hedgehog protein.

21. The method of claim 18, wherein the polypeptide includes at least a portion of the hedgehog protein corresponding to a 19kd fragment of an extracellular domain of the hedgehog protein.

22. The method of claim 18, wherein the hedgehog protein is encoded by a gene of a vertebrate organism.

23. The method of claim 18, wherein the polypeptide includes a *hedgehog* polypeptide sequence represented in the general formula of SEQ ID No. 21.

24. The method of claim 18, wherein the polypeptide includes a *hedgehog* polypeptide sequence represented in the general formula of SEQ ID No. 22.

25. The method of claim 18, wherein the hedgehog protein is encoded by a human *hedgehog* gene.

26. The method of claim 18, wherein the *hedgehog* polypeptide sequence is at least 60 percent identical to an amino acid sequence of a *hedgehog* protein selected from the group consisting of SEQ ID No:9, SEQ ID No:10, SEQ ID No:11, SEQ ID No:12, SEQ ID No:13, SEQ ID No:14, SEQ ID No:15 and SEQ ID No:16.

27. The method of claim 26, wherein the *hedgehog* polypeptide sequence is at least 75 percent identical to an amino acid sequence of a *hedgehog* protein selected from the group consisting of SEQ ID No:9, SEQ ID No:10, SEQ ID No:11, SEQ ID No:12, SEQ ID No:13, SEQ ID No:14, SEQ ID No:15 and SEQ ID No:16.

28. The method of claim 26, wherein the *hedgehog* polypeptide sequence is at least 85 percent identical to an amino acid sequence of a *hedgehog* protein selected from the group consisting of SEQ ID No:9, SEQ ID No:10, SEQ ID No:11, SEQ ID No:12, SEQ ID No:13, SEQ ID No:14, SEQ ID No:15 and SEQ ID No:16.

29. The method of claim 26, wherein the *hedgehog* polypeptide sequence is encodable by a nucleotide sequence which hybridizes under stringent conditions to a sequence selected from the group consisting of SEQ ID No:1, SEQ ID No:2, SEQ ID No:3, SEQ ID No:4, SEQ ID No:5, SEQ ID No:6, SEQ ID No:7 and SEQ ID No:8.

30. The method of claim 23, wherein the *hedgehog* polypeptide sequence is an amino acid sequence of a *hedgehog* protein selected from the group consisting of SEQ ID No:9, SEQ ID No:10, SEQ ID No:11, SEQ ID No:12, SEQ ID No:13, SEQ ID No:14, SEQ ID No:15 and SEQ ID No:16.

31. The method of claim 18, wherein the *hedgehog* polypeptide sequence is an amino acid sequence of a Sonic *hedgehog* protein.

32. The method of claim 18, wherein the *hedgehog* polypeptide sequence is an amino acid sequence of an Indian *hedgehog* protein.

33. The method of claim 18, wherein the *hedgehog* polypeptide sequence is an amino acid sequence of a Desert *hedgehog* protein.

34. The method of claim 18, wherein the *hedgehog* polypeptide sequence includes an amino acid sequence corresponding approximately to residues 24-193 of SEQ ID No:15.

35. The method of claim 18, wherein the polypeptide is purified to at least 80% by dry weight.

36. The method of claim 18, wherein the polypeptide is a recombinantly produced polypeptide.

37. The method of claim 18, wherein the polypeptide is a chemically synthesized polypeptide.

38. The method of claim 17, wherein the hedgehog therapeutic is a peptidomimetic of a *hedgehog* polypeptide sequence.

39. The method of claim 1, 2, 3 or 4, wherein the agent is a ptc therapeutic.

40. The method of claim 39, wherein the ptc therapeutic is a small organic molecule which binds to a *patched* protein and derepresses *patched*-mediated inhibition of mitosis.

41. The method of claims 39, wherein the *ptc* therapeutic binds to *patched* and mimics *hedgehog*-mediated *patched* signal transduction.

42. The method of claim 41, wherein the *ptc* therapeutic is a small organic molecule.

43. The method of claim 41, wherein the binding of the ptc therapeutic to *patched* results in upregulation of patched and/or gli expression.

44. The method of claim 39, wherein the *ptc* therapeutic is a small organic molecule which interacts with epithelial cells to induce *hedgehog*-mediated *patched* signal transduction.

45. The method of claim 44, wherein the *ptc* therapeutic induces *hedgehog*-mediated *patched* signal transduction by altering the localization, protein-protein binding and/or enzymatic activity of an intracellular protein involved in a *patched* signal pathway.

46. The method of claim 39, wherein the *ptc* therapeutic alters the level of expression of a *hedgehog* protein, a patched protein or a protein involved in the intracellular signal transduction pathway of *patched*.

47. The method of claim 46, wherein the *ptc* therapeutic is an antisense construct which inhibits the expression of a protein which is involved in the signal transduction pathway of *patched* and the expression of which antagonizes *hedgehog*-mediated signals.

48. The method of claim 47, wherein the antisense construct is an oligonucleotide of about 20-30 nucleotides in length and having a GC content of at least 50 percent.

49. The method of claim 48, wherein the antisense oligonucleotide is selected from the group consisting of: 5'-GTCCTGGCGCCGCCGCCGCCGTCGCC;

5'-TTCCGATGACCGGCCTTTCGCGGTGA; and

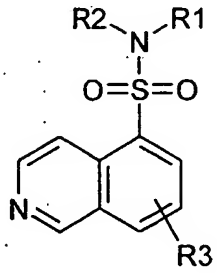
5'-GTGCACGGAAAGGTGCAGGCCACACT

50. The method of claims 44, wherein the *ptc* therapeutic is a small organic molecule which binds to *patched* and regulates *patched*-dependent gene expression.

51. The method of claim 44, wherein the *ptc* therapeutic is an inhibitor of protein kinase A (PKA).

52. The method of claim 51, wherein the PKA inhibitor is a 5-isoquinolinesulfonamide

53. The method of claim 51, wherein the PKA inhibitor is represented in the general formula:



wherein,

R_1 and R_2 each can independently represent hydrogen, and as valence and stability permit a lower alkyl, a lower alkenyl, a lower alkynyl, a carbonyl (such as a carboxyl, an ester, a formate, or a ketone), a thiocarbonyl (such as a thioester, a thioacetate, or a thioformate), an amino, an acylamino, an amido, a cyano, a nitro, an azido, a sulfate, a sulfonate, a

sulfonamido, -$(CH_2)_m$-R_8, -$(CH_2)_m$-OH, -$(CH_2)_m$-O-lower alkyl, -$(CH_2)_m$-O-lower alkenyl, -$(CH_2)_n$-O-$(CH_2)_m$-R_8, -$(CH_2)_m$-SH, -$(CH_2)_m$-S-lower alkyl, -$(CH_2)_m$-S-lower alkenyl, -$(CH_2)_n$-S-$(CH_2)_m$-R_8, or

R_1 and R_2 taken together with N form a heterocycle (substituted or unsubstituted);

R_3 is absent or represents one or more substitutions to the isoquinoline ring such as a lower alkyl, a lower alkenyl, a lower alkynyl, a carbonyl (such as a carboxyl, an ester, a formate, or a ketone), a thiocarbonyl (such as a thioester, a thioacetate, or a thioformate), an amino, an acylamino, an amido, a cyano, a nitro, an azido, a sulfate, a sulfonate, a sulfonamido, -$(CH_2)_m$-R_8, -$(CH_2)_m$-OH, -$(CH_2)_m$-O-lower alkyl, -$(CH_2)_m$-O-lower alkenyl, -$(CH_2)_n$-O-$(CH_2)_m$-R_8, -$(CH_2)_m$-SH, -$(CH_2)_m$-S-lower alkyl, -$(CH_2)_m$-S-lower alkenyl, -$(CH_2)_n$-S-$(CH_2)_m$-R_8;

R_8 represents a substituted or unsubstituted aryl, aralkyl, cycloalkyl, cycloalkenyl, or heterocycle; and

n and m are independently for each occurrence zero or an integer in the range of 1 to 6.

54. The method of claim 51, wherein the PKA inhibitor is cyclic AMP analog.

55. The method of claim 51, wherein the PKA inhibitor is selected from the group consisting of N-[2-((p-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide, 1-(5-isoquinoline-sulfonyl)-2-methylpiperazine, KT5720, 8-bromo-cAMP, dibutyryl-cAMP and PKA Heat Stable Inhibitor isoform α.

56. A method for promoting the proliferation of skin epithelial cells, comprising ectopically contacting the cells with a polypeptide in an amount effective to increase the rate of proliferation of the epithelial cells, which polypeptide comprises an amino acid sequence including at least a bioactive portion of the N-terminal half of a hedgehog protein.

57. The method of claim 56, which method is used as part of a treatment to control a wound healing process.

58. The method of claim 56, wherein the treatment is selected from a group consisting of burn treatment, skin regeneration, skin grafting, pressure sore treatment, dermal ulcer treatment, post surgery scar reduction and treatment of ulcerative colitis.

59. The method of claim 56, which method is used as part of a treatment of alopecia.

60. A method for inhibiting the proliferation of skin epithelial cells, comprising ectopically contacting the cells with agent, in an amount effective to decrease the rate of proliferation of the epithelial cells, which inhibits binding of a hedgehog protein to patched.

61. The method of claim 60, wherein the epithelial cells are hair follicle cells.

62. The method of claim 61, which method inhibits hair growth.

63. A preparation of a polypeptide comprising a hedgehog polypeptide sequence including a bioactive fragment of a *hedgehog* protein, which polypeptide is formulated for topical application to epithelial tissue.

64. The preparation of claim 63, wherein the polypeptide is formulated for topical application to skin.

65. The preparation of claim 63, wherein the polypeptide includes at least 50 amino acids residues of an N-terminal half of the *hedgehog* protein.

66. The preparation of claim 63, wherein the polypeptide includes at least 100 amino acids of an extracellular domain of the hedgehog protein.

67. The preparation of claim 63, wherein the polypeptide includes at least a portion of the hedgehog protein corresponding to a 19kd fragment of an extracellular domain of the hedgehog protein.

68. The preparation of claim 63, wherein the hedgehog protein is encoded by a gene of a vertebrate organism.

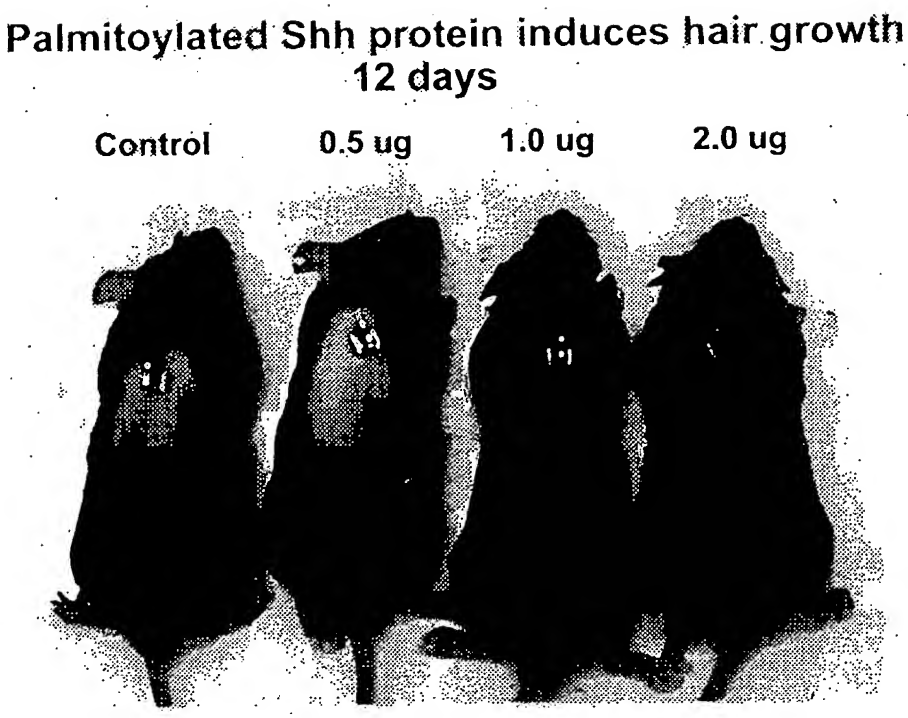

Palmitoylated Shh protein induces hair growth
12 days
Control
0.5 ug
1.0 ug
2.0 ug

Fig. 1A

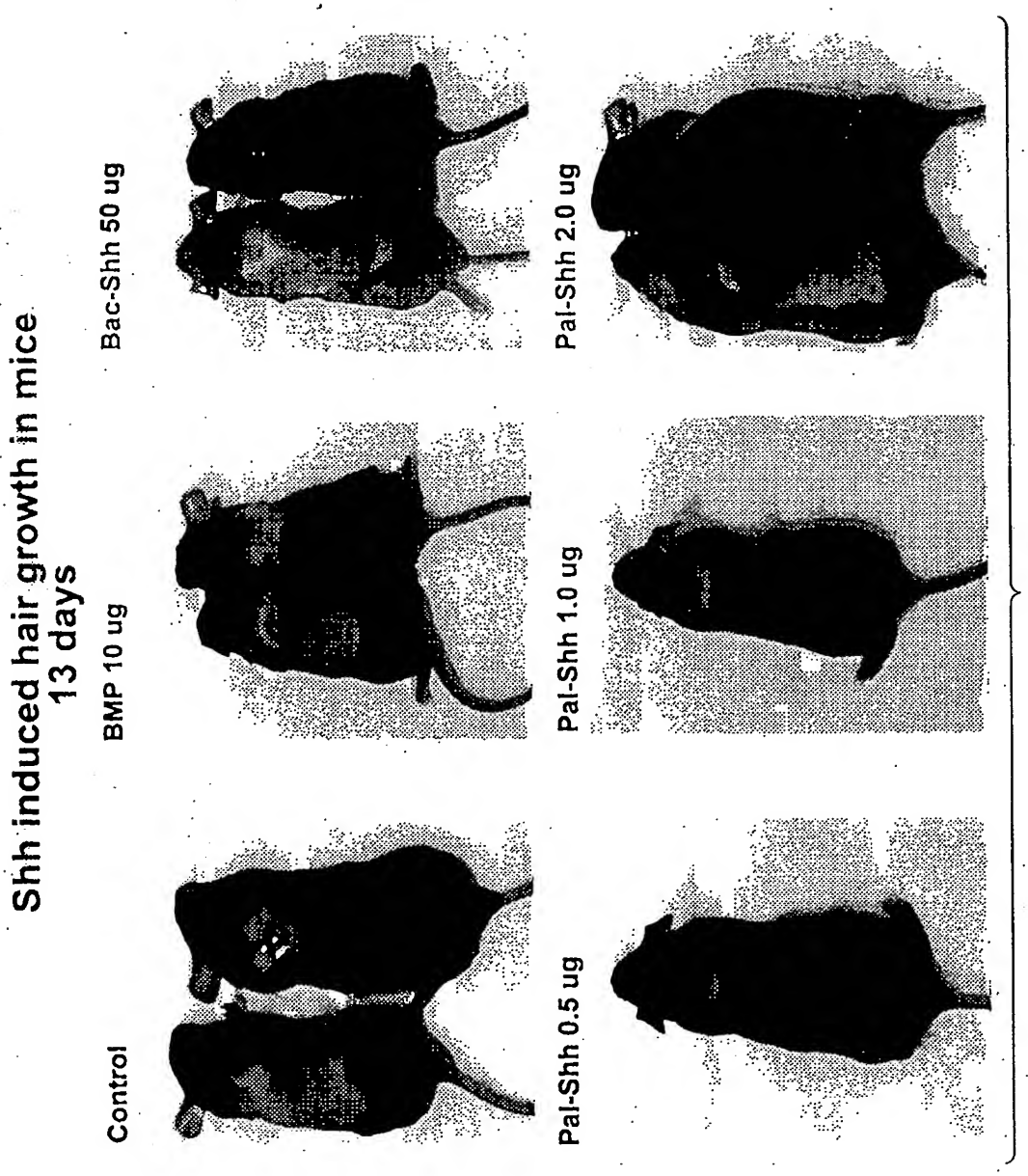

Shh induced hair growth in mice
13 days
Control
BMP 10 ug
Bac-Shh 50 ug
Pal-Shh 0.5 ug
Pal-Shh 1.0 ug
Pal-Shh 2.0 ug

Fig. 1B

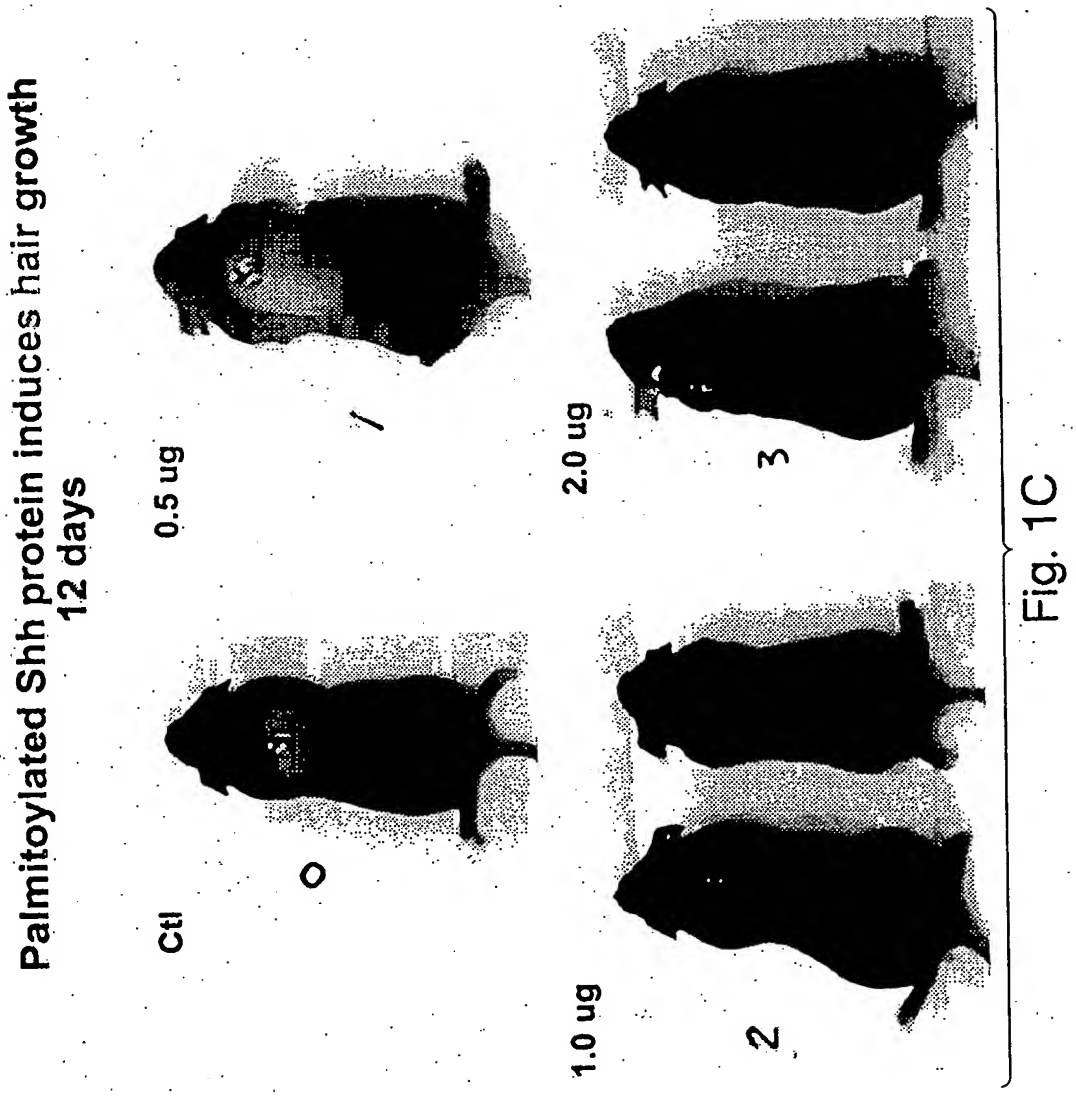

Palmitoylated Shh protein induces hair growth
12 days
Ctl
0
0.5 ug
1
2.0 ug
3
1.0 ug
2

Fig. 1C

SEQUENCE LISTING

(2) INFORMATION FOR SEQ ID NO:1:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1277 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..1275

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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ATG GTC GAA ATG CTG CTG TTG ACA AGA ATT CTC TTG GTG GGC TTC ATC      48
Met Val Glu Met Leu Leu Leu Thr Arg Ile Leu Leu Val Gly Phe Ile
 1               5                  10                  15

TGC GCT CTT TTA GTC TCC TCT GGG CTG ACT TGT GGA CCA GGC AGG GGC      96
Cys Ala Leu Leu Val Ser Ser Gly Leu Thr Cys Gly Pro Gly Arg Gly
            20                  25                  30

ATT GGA AAA AGG AGG CAC CCC AAA AAG CTG ACC CCG TTA GCC TAT AAG     144
Ile Gly Lys Arg Arg His Pro Lys Lys Leu Thr Pro Leu Ala Tyr Lys
        35                  40                  45

CAG TTT ATT CCC AAT GTG GCA GAG AAG ACC CTA GGG GCC AGT GGA AGA     192
Gln Phe Ile Pro Asn Val Ala Glu Lys Thr Leu Gly Ala Ser Gly Arg
    50                  55                  60

TAT GAA GGG AAG ATC ACA AGA AAC TCC GAG AGA TTT AAA GAA CTA ACC     240
Tyr Glu Gly Lys Ile Thr Arg Asn Ser Glu Arg Phe Lys Glu Leu Thr
65                  70                  75                  80

CCA AAT TAC AAC CCT GAC ATT ATT TTT AAG GAT GAA GAG AAC ACG GGA     288
Pro Asn Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Thr Gly
                85                  90                  95

GCT GAC AGA CTG ATG ACT CAG CGC TGC AAG GAC AAG CTG AAT GCC CTG     336
Ala Asp Arg Leu Met Thr Gln Arg Cys Lys Asp Lys Leu Asn Ala Leu
            100                 105                 110

GCG ATC TCG GTG ATG AAC CAG TGG CCC GGG GTG AAG CTG CGG GTG ACC     384
Ala Ile Ser Val Met Asn Gln Trp Pro Gly Val Lys Leu Arg Val Thr
        115                 120                 125

GAG GGC TGG GAC GAG GAT GGC CAT CAC TCC GAG GAA TCG CTG CAC TAC     432
Glu Gly Trp Asp Glu Asp Gly His His Ser Glu Glu Ser Leu His Tyr
    130                 135                 140

GAG GGT CGC GCC GTG GAC ATC ACC ACG TCG GAT CGG GAC CGC AGC AAG     480
Glu Gly Arg Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Arg Ser Lys
145                 150                 155                 160

TAC GGA ATG CTG GCC CGC CTC GCC GTC GAG GCC GGC TTC GAC TGG GTC     528
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Tyr Gly Met Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val
                165                 170                 175

TAC TAC GAG TCC AAG GCG CAC ATC CAC TGC TCC GTC AAA GCA GAA AAC      576
Tyr Tyr Glu Ser Lys Ala His Ile His Cys Ser Val Lys Ala Glu Asn
            180                 185                 190

TCA GTG GCA GCG AAA TCA GGA GGC TGC TTC CCT GGC TCA GCC ACA GTG      624
Ser Val Ala Ala Lys Ser Gly Gly Cys Phe Pro Gly Ser Ala Thr Val
        195                 200                 205

CAC CTG GAG CAT GGA GGC ACC AAG CTG GTG AAG GAC CTG AGC CCT GGG      672
His Leu Glu His Gly Gly Thr Lys Leu Val Lys Asp Leu Ser Pro Gly
    210                 215                 220

GAC CGC GTG CTG GCT GCT GAC GCG GAC GGC CGG CTG CTC TAC AGT GAC      720
Asp Arg Val Leu Ala Ala Asp Ala Asp Gly Arg Leu Leu Tyr Ser Asp
225                 230                 235                 240

TTC CTC ACC TTC CTC GAC CGG ATG GAC AGC TCC CGA AAG CTC TTC TAC      768
Phe Leu Thr Phe Leu Asp Arg Met Asp Ser Ser Arg Lys Leu Phe Tyr
                245                 250                 255

GTC ATC GAG ACG CGG CAG CCC CGG GCC CGG CTG CTA CTG ACG GCG GCC      816
Val Ile Glu Thr Arg Gln Pro Arg Ala Arg Leu Leu Leu Thr Ala Ala
            260                 265                 270

CAC CTG CTC TTT GTG GCC CCC CAG CAC AAC CAG TCG GAG GCC ACA GGG      864
His Leu Leu Phe Val Ala Pro Gln His Asn Gln Ser Glu Ala Thr Gly
        275                 280                 285

TCC ACC AGT GGC CAG GCG CTC TTC GCC AGC AAC GTG AAG CCT GGC CAA      912
Ser Thr Ser Gly Gln Ala Leu Phe Ala Ser Asn Val Lys Pro Gly Gln
    290                 295                 300

CGT GTC TAT GTG CTG GGC GAG GGC GGG CAG CAG CTG CTG CCG GCG TCT      960
Arg Val Tyr Val Leu Gly Glu Gly Gly Gln Gln Leu Leu Pro Ala Ser
305                 310                 315                 320

GTC CAC AGC GTC TCA TTG CGG GAG GAG GCG TCC GGA GCC TAC GCC CCA     1008
Val His Ser Val Ser Leu Arg Glu Glu Ala Ser Gly Ala Tyr Ala Pro
                325                 330                 335

CTC ACC GCC CAG GGC ACC ATC CTC ATC AAC CGG GTG TTG GCC TCC TGC     1056
Leu Thr Ala Gln Gly Thr Ile Leu Ile Asn Arg Val Leu Ala Ser Cys
            340                 345                 350

TAC GCC GTC ATC GAG GAG CAC AGT TGG GCC CAT TGG GCC TTC GCA CCA     1104
Tyr Ala Val Ile Glu Glu His Ser Trp Ala His Trp Ala Phe Ala Pro
        355                 360                 365

TTC CGC TTG GCT CAG GGG CTG CTG GCC GCC CTC TGC CCA GAT GGG GCC     1152
Phe Arg Leu Ala Gln Gly Leu Leu Ala Ala Leu Cys Pro Asp Gly Ala
    370                 375                 380

ATC CCT ACT GCC GCC ACC ACC ACC ACT GGC ATC CAT TGG TAC TCA CGG     1200
Ile Pro Thr Ala Ala Thr Thr Thr Thr Gly Ile His Trp Tyr Ser Arg
385                 390                 395                 400
CTC CTC TAC CGC ATC GGC AGC TGG GTG CTG GAT GGT GAC GCG CTG CAT     1248
Leu Leu Tyr Arg Ile Gly Ser Trp Val Leu Asp Gly Asp Ala Leu His
                405                 410                 415
```

```
CCG CTG GGC ATG GTG GCA CCG GCC AGC TG                                  1277
Pro Leu Gly Met Val Ala Pro Ala Ser
            420                 425
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1190 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..1191

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
ATG GCT CTG CCG GCC AGT CTG TTG CCC CTG TGC TGC TTG GCA CTC TTG          48
Met Ala Leu Pro Ala Ser Leu Leu Pro Leu Cys Cys Leu Ala Leu Leu
  1               5                  10                  15

GCA CTA TCT GCC CAG AGC TGC GGG CCG GGC CGA GGA CCG GTT GGC CGG          96
Ala Leu Ser Ala Gln Ser Cys Gly Pro Gly Arg Gly Pro Val Gly Arg
            20                  25                  30

CGG CGT TAT GTG CGC AAG CAA CTT GTG CCT CTG CTA TAC AAG CAG TTT         144
Arg Arg Tyr Val Arg Lys Gln Leu Val Pro Leu Leu Tyr Lys Gln Phe
        35                  40                  45

GTG CCC AGT ATG CCC GAG CGG ACC CTG GGC GCG AGT GGG CCA GCG GAG         192
Val Pro Ser Met Pro Glu Arg Thr Leu Gly Ala Ser Gly Pro Ala Glu
    50                  55                  60

GGG AGG GTA ACA AGG GGG TCG GAG CGC TTC CGG GAC CTC GTA CCC AAC         240
Gly Arg Val Thr Arg Gly Ser Glu Arg Phe Arg Asp Leu Val Pro Asn
65                  70                  75                  80

TAC AAC CCC GAC ATA ATC TTC AAG GAT GAG GAG AAC AGC GGC GCA GAC         288
Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Ser Gly Ala Asp
                85                  90                  95

CGC CTG ATG ACA GAG CGT TGC AAA GAG CGG GTG AAC GCT CTA GCC ATC         336
Arg Leu Met Thr Glu Arg Cys Lys Glu Arg Val Asn Ala Leu Ala Ile
            100                 105                 110

GCG GTG ATG AAC ATG TGG CCC GGA GTA CGC CTA CGT GTG ACT GAA GGC         384
Ala Val Met Asn Met Trp Pro Gly Val Arg Leu Arg Val Thr Glu Gly
        115                 120                 125

TGG GAC GAG GAC GGC CAC CAC GCA CAG GAT TCA CTC CAC TAC GAA GGC         432
Trp Asp Glu Asp Gly His His Ala Gln Asp Ser Leu His Tyr Glu Gly
    130                 135                 140

CGT GCC TTG GAC ATC ACC ACG TCT GAC CGT GAC CGT AAT AAG TAT GGT         480
Arg Ala Leu Asp Ile Thr Thr Ser Asp Arg Asp Arg Asn Lys Tyr Gly
```

```
145                 150                 155                 160

TTG TTG GCG CGC CTA GCT GTG GAA GCC GGA TTC GAC TGG GTC TAC TAC      528
Leu Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val Tyr Tyr
                165                 170                 175

GAG TCC CGC AAC CAC ATC CAC GTA TCG GTC AAA GCT GAT AAC TCA CTG      576
Glu Ser Arg Asn His Ile His Val Ser Val Lys Ala Asp Asn Ser Leu
            180                 185                 190

GCG GTC CGA GCC GGA GGC TGC TTT CCG GGA AAT GCC ACG GTG CGC TTG      624
Ala Val Arg Ala Gly Gly Cys Phe Pro Gly Asn Ala Thr Val Arg Leu
        195                 200                 205

CGG AGC GGC GAA CGG AAG GGG CTG AGG GAA CTA CAT CGT GGT GAC TGG      672
Arg Ser Gly Glu Arg Lys Gly Leu Arg Glu Leu His Arg Gly Asp Trp
    210                 215                 220

GTA CTG GCC GCT GAT GCA GCG GGC CGA GTG GTA CCC ACG CCA GTG CTG      720
Val Leu Ala Ala Asp Ala Ala Gly Arg Val Val Pro Thr Pro Val Leu
225                 230                 235                 240

CTC TTC CTG GAC CGG GAT CTG CAG CGC CGC GCC TCG TTC GTG GCT GTG      768
Leu Phe Leu Asp Arg Asp Leu Gln Arg Arg Ala Ser Phe Val Ala Val
                245                 250                 255

GAG ACC GAG CGG CCT CCG CGC AAA CTG TTG CTC ACA CCC TGG CAT CTG      816
Glu Thr Glu Arg Pro Pro Arg Lys Leu Leu Leu Thr Pro Trp His Leu
            260                 265                 270

GTG TTC GCT GCT CGC GGG CCA GCG CCT GCT CCA GGT GAC TTT GCA CCG      864
Val Phe Ala Ala Arg Gly Pro Ala Pro Ala Pro Gly Asp Phe Ala Pro
        275                 280                 285

GTG TTC GCG CGC CGC TTA CGT GCT GGC GAC TCG GTG CTG GCT CCC GGC      912
Val Phe Ala Arg Arg Leu Arg Ala Gly Asp Ser Val Leu Ala Pro Gly
    290                 295                 300

GGG GAC GCG CTC CAG CCG GCG CGC GTA GCC CGC GTG GCG CGC GAG GAA      960
Gly Asp Ala Leu Gln Pro Ala Arg Val Ala Arg Val Ala Arg Glu Glu
305                 310                 315                 320

GCC GTG GGC GTG TTC GCA CCG CTC ACT GCG CAC GGG ACG CTG CTG GTC     1008
Ala Val Gly Val Phe Ala Pro Leu Thr Ala His Gly Thr Leu Leu Val
                325                 330                 335

AAC GAC GTC CTC GCC TCC TGC TAC GCG GTT CTA GAG AGT CAC CAG TGG     1056
Asn Asp Val Leu Ala Ser Cys Tyr Ala Val Leu Glu Ser His Gln Trp
            340                 345                 350

GCC CAC CGC GCC TTC GCC CCT TTG CGG CTG CTG CAC GCG CTC GGG GCT     1104
Ala His Arg Ala Phe Ala Pro Leu Arg Leu Leu His Ala Leu Gly Ala
        355                 360                 365

CTG CTC CCT GGG GGT GCA GTC CAG CCG ACT GGC ATG CAT TGG TAC TCT     1152
Leu Leu Pro Gly Gly Ala Val Gln Pro Thr Gly Met His Trp Tyr Ser
    370                 375                 380

CGC CTC CTT TAC CGC TTG GCC GAG GAG TTA ATG GGC TG                  1190
Arg Leu Leu Tyr Arg Leu Ala Glu Glu Leu Met Gly
385                 390                 395
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1281 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..1233

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
ATG TCT CCC GCC TGG CTC CGG CCC CGA CTG CGG TTC TGT CTG TTC CTG        48
Met Ser Pro Ala Trp Leu Arg Pro Arg Leu Arg Phe Cys Leu Phe Leu
 1               5                  10                  15

CTG CTG CTG CTT CTG GTG CCG GCG GCG CGG GGC TGC GGG CCG GGC CGG        96
Leu Leu Leu Leu Leu Val Pro Ala Ala Arg Gly Cys Gly Pro Gly Arg
            20                  25                  30

GTG GTG GGC AGC CGC CGG AGG CCG CCT CGC AAG CTC GTG CCT CTT GCC       144
Val Val Gly Ser Arg Arg Arg Pro Pro Arg Lys Leu Val Pro Leu Ala
        35                  40                  45

TAC AAG CAG TTC AGC CCC AAC GTG CCG GAG AAG ACC CTG GGC GCC AGC       192
Tyr Lys Gln Phe Ser Pro Asn Val Pro Glu Lys Thr Leu Gly Ala Ser
    50                  55                  60

GGG CGC TAC GAA GGC AAG ATC GCG CGC AGC TCT GAG CGC TTC AAA GAG       240
Gly Arg Tyr Glu Gly Lys Ile Ala Arg Ser Ser Glu Arg Phe Lys Glu
65                  70                  75                  80

CTC ACC CCC AAC TAC AAT CCC GAC ATC ATC TTC AAG GAC GAG GAG AAC       288
Leu Thr Pro Asn Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn
                85                  90                  95

ACG GGT GCC GAC CGC CTC ATG ACC CAG CGC TGC AAG GAC CGT CTG AAC       336
Thr Gly Ala Asp Arg Leu Met Thr Gln Arg Cys Lys Asp Arg Leu Asn
            100                 105                 110

TCA CTG GCC ATC TCT GTC ATG AAC CAG TGG CCT GGT GTG AAA CTG CGG       384
Ser Leu Ala Ile Ser Val Met Asn Gln Trp Pro Gly Val Lys Leu Arg
        115                 120                 125

GTG ACC GAA GGC CGG GAT GAA GAT GGC CAT CAC TCA GAG GAG TCT TTA       432
Val Thr Glu Gly Arg Asp Glu Asp Gly His His Ser Glu Glu Ser Leu
    130                 135                 140

CAC TAT GAG GGC CGC GCG GTG GAT ATC ACC ACC TCA GAC CGT GAC CGA       480
His Tyr Glu Gly Arg Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Arg
145                 150                 155                 160

AAT AAG TAT GGA CTG CTG GCG CGC TTA GCA GTG GAG GCC GGC TTC GAC       528
Asn Lys Tyr Gly Leu Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp
                165                 170                 175

TGG GTG TAT TAC GAG TCC AAG GCC CAC GTG CAT TGC TCT GTC AAG TCT       576
```

```
Trp Val Tyr Tyr Glu Ser Lys Ala His Val His Cys Ser Val Lys Ser
            180                 185                 190

GAG CAT TCG GCC GCT GCC AAG ACA GGT GGC TGC TTT CCT GCC GGA GCC      624
Glu His Ser Ala Ala Ala Lys Thr Gly Gly Cys Phe Pro Ala Gly Ala
        195                 200                 205

CAG GTG CGC CTA GAG AAC GGG GAG CGT GTG GCC CTG TCA GCT GTA AAG      672
Gln Val Arg Leu Glu Asn Gly Glu Arg Val Ala Leu Ser Ala Val Lys
    210                 215                 220

CCA GGA GAC CGG GTG CTG GCC ATG GGG GAG GAT GGG ACC CCC ACC TTC      720
Pro Gly Asp Arg Val Leu Ala Met Gly Glu Asp Gly Thr Pro Thr Phe
225                 230                 235                 240

AGT GAT GTG CTT ATT TTC CTG GAC CGC GAG CCA AAC CGG CTG AGA GCT      768
Ser Asp Val Leu Ile Phe Leu Asp Arg Glu Pro Asn Arg Leu Arg Ala
                245                 250                 255

TTC CAG GTC ATC GAG ACT CAG GAT CCT CCG CGT CGG CTG GCG CTC ACG      816
Phe Gln Val Ile Glu Thr Gln Asp Pro Pro Arg Arg Leu Ala Leu Thr
            260                 265                 270

CCT GCC CAC CTG CTC TTC ATT GCG GAC AAT CAT ACA GAA CCA GCA GCC      864
Pro Ala His Leu Leu Phe Ile Ala Asp Asn His Thr Glu Pro Ala Ala
        275                 280                 285

CAC TTC CGG GCC ACA TTT GCC AGC CAT GTG CAA CCA GGC CAA TAT GTG      912
His Phe Arg Ala Thr Phe Ala Ser His Val Gln Pro Gly Gln Tyr Val
    290                 295                 300

CTG GTA TCA GGG GTA CCA GGC CTC CAG CCT GCT CGG GTG GCA GCT GTC      960
Leu Val Ser Gly Val Pro Gly Leu Gln Pro Ala Arg Val Ala Ala Val
305                 310                 315                 320

TCC ACC CAC GTG GCC CTT GGG TCC TAT GCT CCT CTC ACA AGG CAT GGG     1008
Ser Thr His Val Ala Leu Gly Ser Tyr Ala Pro Leu Thr Arg His Gly
                325                 330                 335

ACA CTT GTG GTG GAG GAT GTG GTG GCC TCC TGC TTT GCA GCT GTG GCT     1056
Thr Leu Val Val Glu Asp Val Val Ala Ser Cys Phe Ala Ala Val Ala
            340                 345                 350

GAC CAC CAT CTG GCT CAG TTG GCC TTC TGG CCC CTG CGA CTG TTT CCC     1104
Asp His His Leu Ala Gln Leu Ala Phe Trp Pro Leu Arg Leu Phe Pro
        355                 360                 365

AGT TTG GCA TGG GGC AGC TGG ACC CCA AGT GAG GGT GTT CAC TCC TAC     1152
Ser Leu Ala Trp Gly Ser Trp Thr Pro Ser Glu Gly Val His Ser Tyr
    370                 375                 380

CCT CAG ATG CTC TAC CGC CTG GGG CGT CTC TTG CTA GAA GAG AGC ACC     1200
Pro Gln Met Leu Tyr Arg Leu Gly Arg Leu Leu Leu Glu Glu Ser Thr
385                 390                 395                 400

TTC CAT CCA CTG GGC ATG TCT GGG GCA GGA AGC TGAAGGGACT CTAACCACTG   1253
Phe His Pro Leu Gly Met Ser Gly Ala Gly Ser
                405                 410

CCCTCCTGGA ACTGCTGTGC GTGGATCC                                      1281
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1313 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..1314

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
ATG CTG CTG CTG CTG GCC AGA TGT TTT CTG GTG ATC CTT GCT TCC TCG        48
Met Leu Leu Leu Leu Ala Arg Cys Phe Leu Val Ile Leu Ala Ser Ser
 1               5                  10                  15

CTG CTG GTG TGC CCC GGG CTG GCC TGT GGG CCC GGC AGG GGG TTT GGA        96
Leu Leu Val Cys Pro Gly Leu Ala Cys Gly Pro Gly Arg Gly Phe Gly
            20                  25                  30

AAG AGG CGG CAC CCC AAA AAG CTG ACC CCT TTA GCC TAC AAG CAG TTT       144
Lys Arg Arg His Pro Lys Lys Leu Thr Pro Leu Ala Tyr Lys Gln Phe
        35                  40                  45

ATT CCC AAC GTA GCC GAG AAG ACC CTA GGG GCC AGC GGC AGA TAT GAA       192
Ile Pro Asn Val Ala Glu Lys Thr Leu Gly Ala Ser Gly Arg Tyr Glu
    50                  55                  60

GGG AAG ATC ACA AGA AAC TCC GAA CGA TTT AAG GAA CTC ACC CCC AAT       240
Gly Lys Ile Thr Arg Asn Ser Glu Arg Phe Lys Glu Leu Thr Pro Asn
65                  70                  75                  80

TAC AAC CCC GAC ATC ATA TTT AAG GAT GAG GAA AAC ACG GGA GCA GAC       288
Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Thr Gly Ala Asp
                85                  90                  95

CGG CTG ATC ACT CAG AGG TGC AAA GAC AAG TTA AAT GCC TTG GCC ATC       336
Arg Leu Met Thr Gln Arg Cys Lys Asp Lys Leu Asn Ala Leu Ala Ile
            100                 105                 110

TCT GTG ATG AAC CAG TGG CCT GGA GTG AGG CTG CGA GTG ACC GAG GGC       384
Ser Val Met Asn Gln Trp Pro Gly Val Arg Leu Arg Val Thr Glu Gly
        115                 120                 125

TGG GAT GAG GAC GGC CAT CAT TCA GAG GAG TCT CTA CAC TAT GAG GGT       432
Trp Asp Glu Asp Gly His His Ser Glu Glu Ser Leu His Tyr Glu Gly
    130                 135                 140

CGA GCA GTG GAC ATC ACC ACG TCC GAC CGG GAC CGC AGC AAG TAC GGC       480
Arg Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Arg Ser Lys Tyr Gly
145                 150                 155                 160

ATG CTG GCT CGC CTG GCT GTG GAA GCA GGT TTC GAC TGG GTC TAC TAT       528
Met Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val Tyr Tyr
                165                 170                 175
```

```
GAA TCC AAA GCT CAC ATC CAC TGT TCT GTG AAA GCA GAG AAC TCC GTG      576
Glu Ser Lys Ala His Ile His Cys Ser Val Lys Ala Glu Asn Ser Val
            180                 185                 190

GCG GCC AAA TCC GGC GGC TGT TTC CCG GGA TCC GCC ACC GTG CAC CTG      624
Ala Ala Lys Ser Gly Gly Cys Phe Pro Gly Ser Ala Thr Val His Leu
        195                 200                 205

GAG CAG GGC GGC ACC AAG CTG GTG AAG GAC TTA CGT CCC GGA GAC CGC      672
Glu Gln Gly Gly Thr Lys Leu Val Lys Asp Leu Arg Pro Gly Asp Arg
    210                 215                 220

GTG CTG GCG GCT GAC GAC CAG GGC CGG CTG CTG TAC AGC GAC TTC CTC      720
Val Leu Ala Ala Asp Asp Gln Gly Arg Leu Leu Tyr Ser Asp Phe Leu
225                 230                 235                 240

ACC TTC CTG GAC CGC GAC GAA GGC GCC AAG AAG GTC TTC TAC GTG ATC      768
Thr Phe Leu Asp Arg Asp Glu Gly Ala Lys Lys Val Phe Tyr Val Ile
                245                 250                 255

GAG ACG CTG GAG CCG CGC GAG CGC CTG CTG CTC ACC GCC GCG CAC CTG      816
Glu Thr Leu Glu Pro Arg Glu Arg Leu Leu Leu Thr Ala Ala His Leu
            260                 265                 270

CTC TTC GTG GCG CCG CAC AAC GAC TCG GGG CCC ACG CCC GGG CCA AGC      864
Leu Phe Val Ala Pro His Asn Asp Ser Gly Pro Thr Pro Gly Pro Ser
        275                 280                 285

GCG CTC TTT GCC AGC CGC GTG CGC CCC GGG CAG CGC GTG TAC GTG GTG      912
Ala Leu Phe Ala Ser Arg Val Arg Pro Gly Gln Arg Val Tyr Val Val
    290                 295                 300

GCT GAA CGC GGC GGG GAC CGC CGG CTG CTG CCC GCC GCG GTG CAC AGC      960
Ala Glu Arg Gly Gly Asp Arg Arg Leu Leu Pro Ala Ala Val His Ser
305                 310                 315                 320

GTG ACG CTG CGA GAG GAG GAG GCG GGC GCG TAC GCG CCG CTC ACG GCG     1008
Val Thr Leu Arg Glu Glu Glu Ala Gly Ala Tyr Ala Pro Leu Thr Ala
                325                 330                 335

CAC GGC ACC ATT CTC ATC AAC CGG GTG CTC GCC TCG TGC TAC GCT GTC     1056
His Gly Thr Ile Leu Ile Asn Arg Val Leu Ala Ser Cys Tyr Ala Val
            340                 345                 350

ATC GAG GAG CAC AGC TGG GCA CAC CGG GCC TTC GCG CCT TTC CGC CTG     1104
Ile Glu Glu His Ser Trp Ala His Arg Ala Phe Ala Pro Phe Arg Leu
        355                 360                 365

GCG CAC GCG CTG CTG GCC GCG CTG GCA CCC GCC CGC ACG GAC GGC GGG     1152
Ala His Ala Leu Leu Ala Ala Leu Ala Pro Ala Arg Thr Asp Gly Gly
    370                 375                 380

GGC GGG GGC AGC ATC CCT GCA GCG CAA TCT GCA ACG GAA GCG AGG GGC     1200
Gly Gly Gly Ser Ile Pro Ala Ala Gln Ser Ala Thr Glu Ala Arg Gly
385                 390                 395                 400

GCG GAG CCG ACT GCG GGC ATC CAC TGG TAC TCG CAG CTG CTC TAC CAC     1248
Ala Glu Pro Thr Ala Gly Ile His Trp Tyr Ser Gln Leu Leu Tyr His
                405                 410                 415

ATT GGC ACC TGG CTG TTG GAC AGC GAG ACC ATG CAT CCC TTG GGA ATG     1296
Ile Gly Thr Trp Leu Leu Asp Ser Glu Thr Met His Pro Leu Gly Met
```

```
           420                 425                 430

GCG GTC AAG TCC AGC TG                                              1313
Ala Val Lys Ser Ser
       435
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1256 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..1257

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
ATG CGG CTT TTG ACG AGA GTG CTG CTG GTG TCT CTT CTC ACT CTG TCC      48
Met Arg Leu Leu Thr Arg Val Leu Leu Val Ser Leu Leu Thr Leu Ser
 1               5                  10                  15

TTG GTG GTG TCC GGA CTG GCC TGC GGT CCT GGC AGA GGC TAC GGC AGA      96
Leu Val Val Ser Gly Leu Ala Cys Gly Pro Gly Arg Gly Tyr Gly Arg
            20                  25                  30

AGA AGA CAT CCG AAG AAG CTG ACA CCT CTC GCC TAC AAG CAG TTC ATA     144
Arg Arg His Pro Lys Lys Leu Thr Pro Leu Ala Tyr Lys Gln Phe Ile
        35                  40                  45

CCT AAT GTC GCG GAG AAG ACC TTA GGG GCC AGC GGC AGA TAC GAG GGC     192
Pro Asn Val Ala Glu Lys Thr Leu Gly Ala Ser Gly Arg Tyr Glu Gly
    50                  55                  60

AAG ATA ACG CGC AAT TCG GAG AGA TTT AAA GAA CTT ACT CCA AAT TAC     240
Lys Ile Thr Arg Asn Ser Glu Arg Phe Lys Glu Leu Thr Pro Asn Tyr
65                  70                  75                  80

AAT CCC GAC ATT ATC TTT AAG GAT GAG GAG AAC ACG GGA GCG GAC AGG     288
Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Thr Gly Ala Asp Arg
                85                  90                  95

CTC ATG ACA CAG AGA TGC AAA GAC AAG CTG AAC TCG CTG GCC ATC TCT     336
Leu Met Thr Gln Arg Cys Lys Asp Lys Leu Asn Ser Leu Ala Ile Ser
            100                 105                 110

GTA ATG AAC CAC TGG CCA GGG GTT AAG CTG CGT GTG ACA GAG GGC TGG     384
Val Met Asn His Trp Pro Gly Val Lys Leu Arg Val Thr Glu Gly Trp
        115                 120                 125

GAT GAG GAC GGT CAC CAT TTT GAA GAA TCA CTC CAC TAC GAG GGA AGA     432
Asp Glu Asp Gly His His Phe Glu Glu Ser Leu His Tyr Glu Gly Arg
    130                 135                 140

GCT GTT GAT ATT ACC ACC TCT GAC CGA GAC AAG AGC AAA TAC GGG ACA     480
```

```
Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Lys Ser Lys Tyr Gly Thr
145                 150                 155                 160

CTG TCT CGC CTA GCT GTG GAG GCT GGA TTT GAC TGG GTC TAT TAC GAG      528
Leu Ser Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val Tyr Tyr Glu
                165                 170                 175

TCC AAA GCC CAC ATT CAT TGC TCT GTC AAA GCA GAA AAT TCG GTT GCT      576
Ser Lys Ala His Ile His Cys Ser Val Lys Ala Glu Asn Ser Val Ala
            180                 185                 190

GCG AAA TCT GGG GGC TGT TTC CCA GGT TCG GCT CTG GTC TCG CTC CAG      624
Ala Lys Ser Gly Gly Cys Phe Pro Gly Ser Ala Leu Val Ser Leu Gln
        195                 200                 205

GAC GGA GGA CAG AAG GCC GTG AAG GAC CTG AAC CCC GGA GAC AAG GTG      672
Asp Gly Gly Gln Lys Ala Val Lys Asp Leu Asn Pro Gly Asp Lys Val
    210                 215                 220

CTG GCG GCA GAC AGC GCG GGA AAC CTG GTG TTC AGC GAC TTC ATC ATG      720
Leu Ala Ala Asp Ser Ala Gly Asn Leu Val Phe Ser Asp Phe Ile Met
225                 230                 235                 240

TTC ACA GAC CGA GAC TCC ACG ACG CGA CGT GTG TTT TAC GTC ATA GAA      768
Phe Thr Asp Arg Asp Ser Thr Thr Arg Arg Val Phe Tyr Val Ile Glu
                245                 250                 255

ACG CAA GAA CCC GTT GAA AAG ATC ACC CTC ACC GCC GCT CAC CTC CTT      816
Thr Gln Glu Pro Val Glu Lys Ile Thr Leu Thr Ala Ala His Leu Leu
            260                 265                 270

TTT GTC CTC GAC AAC TCA ACG GAA GAT CTC CAC ACC ATG ACC GCC GCG      864
Phe Val Leu Asp Asn Ser Thr Glu Asp Leu His Thr Met Thr Ala Ala
        275                 280                 285

TAT GCC AGC AGT GTC AGA GCC GGA CAA AAG GTG ATG GTT GTT GAT GAT      912
Tyr Ala Ser Ser Val Arg Ala Gly Gln Lys Val Met Val Val Asp Asp
    290                 295                 300

AGC GGT CAG CTT AAA TCT GTC ATC GTG CAG CGG ATA TAC ACG GAG GAG      960
Ser Gly Gln Leu Lys Ser Val Ile Val Gln Arg Ile Tyr Thr Glu Glu
305                 310                 315                 320

CAG CGG GGC TCG TTC GCA CCA GTG ACT GCA CAT GGG ACC ATT GTG GTC     1008
Gln Arg Gly Ser Phe Ala Pro Val Thr Ala His Gly Thr Ile Val Val
                325                 330                 335

GAC AGA ATA CTG GCG TCC TGT TAC GCC GTA ATA GAG GAC CAG GGG CTT     1056
Asp Arg Ile Leu Ala Ser Cys Tyr Ala Val Ile Glu Asp Gln Gly Leu
            340                 345                 350

GCG CAT TTG GCC TTC GCG CCC GCC AGG CTC TAT TAT TAC GTG TCA TCA     1104
Ala His Leu Ala Phe Ala Pro Ala Arg Leu Tyr Tyr Tyr Val Ser Ser
        355                 360                 365

TTC CTG TCC CCC AAA ACT CCA GCA GTC GGT CCA ATG CGA CTT TAC AAC     1152
Phe Leu Ser Pro Lys Thr Pro Ala Val Gly Pro Met Arg Leu Tyr Asn
    370                 375                 380

AGG AGG GGG TCC ACT GGT ACT CCA GGC TCC TGT CAT CAA ATG GGA ACG     1200
Arg Arg Gly Ser Thr Gly Thr Pro Gly Ser Cys His Gln Met Gly Thr
385                 390                 395                 400
```

```
TGG CTT TTG GAC AGC AAC ATG CTT CAT CCT TTG GGG ATG TCA GTA AAC      1248
Trp Leu Leu Asp Ser Asn Met Leu His Pro Leu Gly Met Ser Val Asn
                405                 410                 415

TCA AGC TG                                                       1256
Ser Ser
```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1425 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..1425

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
ATG CTG CTG CTG GCG AGA TGT CTG CTG CTA GTC CTC GTC TCC TCG CTG        48
Met Leu Leu Leu Ala Arg Cys Leu Leu Leu Val Leu Val Ser Ser Leu
  1               5                  10                  15

CTG GTA TGC TCG GGA CTG GCG TGC GGA CCG GGC AGG GGG TTC GGG AAG        96
Leu Val Cys Ser Gly Leu Ala Cys Gly Pro Gly Arg Gly Phe Gly Lys
            20                  25                  30

AGG AGG CAC CCC AAA AAG CTG ACC CCT TTA GCC TAC AAG CAG TTT ATC       144
Arg Arg His Pro Lys Lys Leu Thr Pro Leu Ala Tyr Lys Gln Phe Ile
        35                  40                  45

CCC AAT GTG GCC GAG AAG ACC CTA GGC GCC AGC GGA AGG TAT GAA GGG       192
Pro Asn Val Ala Glu Lys Thr Leu Gly Ala Ser Gly Arg Tyr Glu Gly
    50                  55                  60

AAG ATC TCC AGA AAC TCC GAG CGA TTT AAG GAA CTC ACC CCC AAT TAC       240
Lys Ile Ser Arg Asn Ser Glu Arg Phe Lys Glu Leu Thr Pro Asn Tyr
65                  70                  75                  80

AAC CCC GAC ATC ATA TTT AAG GAT GAA GAA AAC ACC GGA GCG GAC AGG       288
Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Thr Gly Ala Asp Arg
                85                  90                  95

CTG ATG ACT CAG AGG TGT AAG GAC AAG TTG AAC GCT TTG GCC ATC TCG       336
Leu Met Thr Gln Arg Cys Lys Asp Lys Leu Asn Ala Leu Ala Ile Ser
            100                 105                 110

GTG ATG AAC CAG TGG CCA GGA GTG AAA CTG CGG GTG ACC GAG GGC TGG       384
Val Met Asn Gln Trp Pro Gly Val Lys Leu Arg Val Thr Glu Gly Trp
        115                 120                 125

GAC GAA GAT GGC CAC CAC TCA GAG GAG TCT CTG CAC TAC GAG GGC CGC       432
Asp Glu Asp Gly His His Ser Glu Glu Ser Leu His Tyr Glu Gly Arg
    130                 135                 140
```

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GCA GTG GAC ATC ACC ACG TCT GAC CGC GAC CGC AGC AAG TAC GGC ATG      480
Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Arg Ser Lys Tyr Gly Met
145                 150                 155                 160

CTG GCC CGC CTG GCG GTG GAG GCC GGC TTC GAC TGG GTG TAC TAC GAG      528
Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val Tyr Tyr Glu
                165                 170                 175

TCC AAG GCA CAT ATC CAC TGC TCG GTG AAA GCA GAG AAC TCG GTG GCG      576
Ser Lys Ala His Ile His Cys Ser Val Lys Ala Glu Asn Ser Val Ala
            180                 185                 190

GCC AAA TCG GGA GGC TGC TTC CCG GGC TCG GCC ACG GTG CAC CTG GAG      624
Ala Lys Ser Gly Gly Cys Phe Pro Gly Ser Ala Thr Val His Leu Glu
        195                 200                 205

CAG GGC GGC ACC AAG CTG GTG AAG GAC CTG AGC CCC GGG GAC CGC GTG      672
Gln Gly Gly Thr Lys Leu Val Lys Asp Leu Ser Pro Gly Asp Arg Val
    210                 215                 220

CTG GCG GCG GAC GAC CAG GGC CGG CTG CTC TAC AGC GAC TTC CTC ACT      720
Leu Ala Ala Asp Asp Gln Gly Arg Leu Leu Tyr Ser Asp Phe Leu Thr
225                 230                 235                 240

TTC CTG GAC CGC GAC GAC GGC GCC AAG AAG GTC TTC TAC GTG ATC GAG      768
Phe Leu Asp Arg Asp Asp Gly Ala Lys Lys Val Phe Tyr Val Ile Glu
                245                 250                 255

ACG CGG GAG CCG CGC GAG CGC CTG CTG CTC ACC GCC GCG CAC CTG CTC      816
Thr Arg Glu Pro Arg Glu Arg Leu Leu Leu Thr Ala Ala His Leu Leu
            260                 265                 270

TTT GTG GCG CCG CAC AAC GAC TCG GCC ACC GGG GAG CCC GAG GCG TCC      864
Phe Val Ala Pro His Asn Asp Ser Ala Thr Gly Glu Pro Glu Ala Ser
        275                 280                 285

TCG GGC TCG GGG CCG CCT TCC GGG GGC GCA CTG GGG CCT CGG GCG CTG      912
Ser Gly Ser Gly Pro Pro Ser Gly Gly Ala Leu Gly Pro Arg Ala Leu
    290                 295                 300

TTC GCC AGC CGC GTG CGC CCG GGC CAG CGC GTG TAC GTG GTG GCC GAG      960
Phe Ala Ser Arg Val Arg Pro Gly Gln Arg Val Tyr Val Val Ala Glu
305                 310                 315                 320

CGT GAC GGG GAC CGC CGG CTC CTG CCC GCC GCT GTG CAC AGC GTG ACC     1008
Arg Asp Gly Asp Arg Arg Leu Leu Pro Ala Ala Val His Ser Val Thr
                325                 330                 335

CTA AGC GAG GAG GCC GCG GGC GCC TAC GCG CCG CTC ACG GCC CAG GGC     1056
Leu Ser Glu Glu Ala Ala Gly Ala Tyr Ala Pro Leu Thr Ala Gln Gly
            340                 345                 350

ACC ATT CTC ATC AAC CGG GTG CTG GCC TCG TGC TAC GCG GTC ATC GAG     1104
Thr Ile Leu Ile Asn Arg Val Leu Ala Ser Cys Tyr Ala Val Ile Glu
        355                 360                 365

GAG CAC AGC TGG GCG CAC CGG GCC TTC GCG CCC TTC CGC CTG GCG CAC     1152
Glu His Ser Trp Ala His Arg Ala Phe Ala Pro Phe Arg Leu Ala His
    370                 375                 380

GCG CTC CTG GCT GCA CTG GCG CCC GCG CGC ACG GAC CGC GGC GGG GAC     1200
Ala Leu Leu Ala Ala Leu Ala Pro Ala Arg Thr Asp Arg Gly Gly Asp
```

```
385                 390                 395                 400

AGC GGC GGC GGG GAC CGC GGG GGC GGC GGC GGC AGA GTA GCC CTA ACC      1248
Ser Gly Gly Gly Asp Arg Gly Gly Gly Gly Gly Arg Val Ala Leu Thr
                405                 410                 415

GCT CCA GGT GCT GCC GAC GCT CCG GGT GCG GGG GCC ACC GCG GGC ATC      1296
Ala Pro Gly Ala Ala Asp Ala Pro Gly Ala Gly Ala Thr Ala Gly Ile
            420                 425                 430

CAC TGG TAC TCG CAG CTG CTC TAC CAA ATA GGC ACC TGG CTC CTG GAC      1344
His Trp Tyr Ser Gln Leu Leu Tyr Gln Ile Gly Thr Trp Leu Leu Asp
        435                 440                 445

AGC GAG GCC CTG CAC CCG CTG GGC ATG GCG GTC AAG TCC AGC NNN AGC      1392
Ser Glu Ala Leu His Pro Leu Gly Met Ala Val Lys Ser Ser Xaa Ser
    450                 455                 460

CGG GGG GCC GGG GGA GGG GCG CGG GAG GGG GCC                          1425
Arg Gly Ala Gly Gly Gly Ala Arg Glu Gly Ala
465                 470                 475
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1622 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 51..1283

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

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CATCAGCCCA CCAGGAGACC TCGCCCGCCG CTCCCCCGGG CTCCCCGGCC ATG TCT        56
                                                          Met Ser
                                                            1

CCC GCC CGG CTC CGG CCC CGA CTG CAC TTC TGC CTG GTC CTG TTG CTG       104
Pro Ala Arg Leu Arg Pro Arg Leu His Phe Cys Leu Val Leu Leu Leu
          5                  10                  15

CTG CTG GTG GTG CCC GCG GCA TGG GGC TGC GGG CCG GGT CGG GTG GTG       152
Leu Leu Val Val Pro Ala Ala Trp Gly Cys Gly Pro Gly Arg Val Val
     20                  25                  30

GGC AGC CGC CGG CGA CCG CCA CGC AAA CTC GTG CCG CTC GCC TAC AAG       200
Gly Ser Arg Arg Arg Pro Pro Arg Lys Leu Val Pro Leu Ala Tyr Lys
 35                  40                  45                  50

CAG TTC AGC CCC AAT GTG CCC GAG AAG ACC CTG GGC GCC AGC GGA CGC       248
Gln Phe Ser Pro Asn Val Pro Glu Lys Thr Leu Gly Ala Ser Gly Arg
                 55                  60                  65

TAT GAA GGC AAG ATC GCT CGC AGC TCC GAG CGC TTC AAG GAG CTC ACC       296
```

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Tyr Glu Gly Lys Ile Ala Arg Ser Ser Glu Arg Phe Lys Glu Leu Thr
                70                  75                  80

CCC AAT TAC AAT CCA GAC ATC ATC TTC AAG GAC GAG GAG AAC ACA GGC      344
Pro Asn Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Thr Gly
         85                  90                  95

GCC GAC CGC CTC ATG ACC CAG CGC TGC AAG GAC CGC CTG AAC TCG CTG      392
Ala Asp Arg Leu Met Thr Gln Arg Cys Lys Asp Arg Leu Asn Ser Leu
    100                 105                 110

GCT ATC TCG GTG ATG AAC CAG TGG CCC GGT GTG AAG CTG CGG GTG ACC      440
Ala Ile Ser Val Met Asn Gln Trp Pro Gly Val Lys Leu Arg Val Thr
115                 120                 125                 130

GAG GGC TGG GAC GAG GAC GGC CAC CAC TCA GAG GAG TCC CTG CAT TAT      488
Glu Gly Trp Asp Glu Asp Gly His His Ser Glu Glu Ser Leu His Tyr
                135                 140                 145

GAG GGC CGC GCG GTG GAC ATC ACC ACA TCA GAC CGC GAC CGC AAT AAG      536
Glu Gly Arg Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Arg Asn Lys
            150                 155                 160

TAT GGA CTG CTG GCG CGC TTG GCA GTG GAG GCC GGC TTT GAC TGG GTG      584
Tyr Gly Leu Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val
        165                 170                 175

TAT TAC GAG TCA AAG GCC CAC GTG CAT TGC TCC GTC AAG TCC GAG CAC      632
Tyr Tyr Glu Ser Lys Ala His Val His Cys Ser Val Lys Ser Glu His
    180                 185                 190

TCG GCC GCA GCC AAG ACG GGC GGC TGC TTC CCT GCC GGA GCC CAG GTA      680
Ser Ala Ala Ala Lys Thr Gly Gly Cys Phe Pro Ala Gly Ala Gln Val
195                 200                 205                 210

CGC CTG GAG AGT GGG GCG CGT GTG GCC TTG TCA GCC GTG AGG CCG GGA      728
Arg Leu Glu Ser Gly Ala Arg Val Ala Leu Ser Ala Val Arg Pro Gly
                215                 220                 225

GAC CGT GTG CTG GCC ATG GGG GAG GAT GGG AGC CCC ACC TTC AGC GAT      776
Asp Arg Val Leu Ala Met Gly Glu Asp Gly Ser Pro Thr Phe Ser Asp
            230                 235                 240

GTG CTC ATT TTC CTG GAC CGC GAG CCC CAC AGG CTG AGA GCC TTC CAG      824
Val Leu Ile Phe Leu Asp Arg Glu Pro His Arg Leu Arg Ala Phe Gln
        245                 250                 255

GTC ATC GAG ACT CAG GAC CCC CCA CGC CGC CTG GCA CTC ACA CCC GCT      872
Val Ile Glu Thr Gln Asp Pro Pro Arg Arg Leu Ala Leu Thr Pro Ala
    260                 265                 270

CAC CTG CTC TTT ACG GCT GAC AAT CAC ACG GAG CCG GCA GCC CGC TTC      920
His Leu Leu Phe Thr Ala Asp Asn His Thr Glu Pro Ala Ala Arg Phe
275                 280                 285                 290

CGG GCC ACA TTT GCC AGC CAC GTG CAG CCT GGC CAG TAC GTG CTG GTG      968
Arg Ala Thr Phe Ala Ser His Val Gln Pro Gly Gln Tyr Val Leu Val
                295                 300                 305

GCT GGG GTG CCA GGC CTG CAG CCT GCC CGC GTG GCA GCT GTC TCT ACA     1016
Ala Gly Val Pro Gly Leu Gln Pro Ala Arg Val Ala Ala Val Ser Thr
            310                 315                 320
```

```
CAC GTG GCC CTC GGG GCC TAC GCC CCG CTC ACA AAG CAT GGG ACA CTG      1064
His Val Ala Leu Gly Ala Tyr Ala Pro Leu Thr Lys His Gly Thr Leu
      325                 330                 335

GTG GTG GAG GAT GTG GTG GCA TCC TGC TTC GCG GCC GTG GCT GAC CAC      1112
Val Val Glu Asp Val Val Ala Ser Cys Phe Ala Ala Val Ala Asp His
    340                 345                 350

CAC CTG GCT CAG TTG GCC TTC TGG CCC CTG AGA CTC TTT CAC AGC TTG      1160
His Leu Ala Gln Leu Ala Phe Trp Pro Leu Arg Leu Phe His Ser Leu
355                 360                 365                 370

GCA TGG GGC AGC TGG ACC CCG GGG GAG GGT GTG CAT TGG TAC CCC CAG      1208
Ala Trp Gly Ser Trp Thr Pro Gly Glu Gly Val His Trp Tyr Pro Gln
                375                 380                 385

CTG CTC TAC CGC CTG GGG CGT CTC CTG CTA GAA GAG GGC AGC TTC CAC      1256
Leu Leu Tyr Arg Leu Gly Arg Leu Leu Leu Glu Glu Gly Ser Phe His
          390                 395                 400

CCA CTG GGC ATG TCC GGG GCA GGG AGC TGAAAGGACT CCACCGCTGC            1303
Pro Leu Gly Met Ser Gly Ala Gly Ser
       405                 410

CCTCCTGGAA CTGCTGTACT GGGTCCAGAA GCCTCTCAGC CAGGAGGGAG CTGGCCCTGG    1363

AAGGGACCTG AGCTGGGGGA CACTGGCTCC TGCCATCTCC TCTGCCATGA AGATACACCA    1423

TTGAGACTTG ACTGGGCAAC ACCAGCGTCC CCCACCCGCG TCGTGGTGTA GTCATAGAGC    1483

TGCAAGCTGA GCTGGCGAGG GGATGGTTGT TGACCCCTCT CTCCTAGAGA CCTTGAGGCT    1543

GGCACGGCGA CTCCCAACTC AGCCTGCTCT CACTACGAGT TTTCATACTC TGCCTCCCCC    1603

ATTGGGAGGG CCCATTCCC                                                 1622
```

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1191 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..1191

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```
ATG GCT CTC CTG ACC AAT CTA CTG CCC TTG TGC TGC TTG GCA CTT CTG       48
Met Ala Leu Leu Thr Asn Leu Leu Pro Leu Cys Cys Leu Ala Leu Leu
  1               5                  10                  15

GCG CTG CCA GCC CAG AGC TGC GGG CCG GGC CGG GGG CCG GTT GGC CGG       96
Ala Leu Pro Ala Gln Ser Cys Gly Pro Gly Arg Gly Pro Val Gly Arg
            20                  25                  30
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CGC CGC TAT GCG CGC AAG CAG CTC GTG CCG CTA CTC TAC AAG CAA TTT      144
Arg Arg Tyr Ala Arg Lys Gln Leu Val Pro Leu Leu Tyr Lys Gln Phe
        35                  40                  45

GTG CCC GGC GTG CCA GAG CGG ACC CTG GGC GCC AGT GGG CCA GCG GAG      192
Val Pro Gly Val Pro Glu Arg Thr Leu Gly Ala Ser Gly Pro Ala Glu
    50                  55                  60

GGG AGG GTG GCA AGG GGC TCC GAG CGC TTC CGG GAC CTC GTG CCC AAC      240
Gly Arg Val Ala Arg Gly Ser Glu Arg Phe Arg Asp Leu Val Pro Asn
65                  70                  75                  80

TAC AAC CCC GAC ATC ATC TTC AAG GAT GAG GAG AAC AGT GGA GCC GAC      288
Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Ser Gly Ala Asp
                85                  90                  95

CGC CTG ATG ACC GAG CGT TGC AAG GAG AGG GTG AAC GCT TTG GCC ATT      336
Arg Leu Met Thr Glu Arg Cys Lys Glu Arg Val Asn Ala Leu Ala Ile
            100                 105                 110

GCC GTG ATG AAC ATG TGG CCC GGA GTG CGC CTA CGA GTG ACT GAG GGC      384
Ala Val Met Asn Met Trp Pro Gly Val Arg Leu Arg Val Thr Glu Gly
        115                 120                 125

TGG GAC GAG GAC GGC CAC CAC GCT CAG GAT TCA CTC CAC TAC GAA GGC      432
Trp Asp Glu Asp Gly His His Ala Gln Asp Ser Leu His Tyr Glu Gly
    130                 135                 140

CGT GCT TTG GAC ATC ACT ACG TCT GAC CGC GAC CGC AAC AAG TAT GGG      480
Arg Ala Leu Asp Ile Thr Thr Ser Asp Arg Asp Arg Asn Lys Tyr Gly
145                 150                 155                 160

TTG CTG GCG CGC CTC GCA GTG GAA GCC GGC TTC GAC TGG GTC TAC TAC      528
Leu Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val Tyr Tyr
                165                 170                 175

GAG TCC CGC AAC CAC GTC CAC GTG TCG GTC AAA GCT GAT AAC TCA CTG      576
Glu Ser Arg Asn His Val His Val Ser Val Lys Ala Asp Asn Ser Leu
            180                 185                 190

GCG GTC CGG GCG GGC GGC TGC TTT CCG GGA AAT GCA ACT GTG CGC CTG      624
Ala Val Arg Ala Gly Gly Cys Phe Pro Gly Asn Ala Thr Val Arg Leu
        195                 200                 205

TGG AGC GGC GAG CGG AAA GGG CTG CGG GAA CTG CAC CGC GGA GAC TGG      672
Trp Ser Gly Glu Arg Lys Gly Leu Arg Glu Leu His Arg Gly Asp Trp
    210                 215                 220

GTT TTG GCG GCC GAT GCG TCA GGC CGG GTG GTG CCC ACG CCG GTG CTG      720
Val Leu Ala Ala Asp Ala Ser Gly Arg Val Val Pro Thr Pro Val Leu
225                 230                 235                 240

CTC TTC CTG GAC CGG GAC TTG CAG CGC CGG GCT TCA TTT GTG GCT GTG      768
Leu Phe Leu Asp Arg Asp Leu Gln Arg Arg Ala Ser Phe Val Ala Val
                245                 250                 255

GAG ACC GAG TGG CCT CCA CGC AAA CTG TTG CTC ACG CCC TGG CAC CTG      816
Glu Thr Glu Trp Pro Pro Arg Lys Leu Leu Leu Thr Pro Trp His Leu
            260                 265                 270

GTG TTT GCC GCT CGA GGG CCG GCG CCC GCG CCA GGC GAC TTT GCA CCG      864
Val Phe Ala Ala Arg Gly Pro Ala Pro Ala Pro Gly Asp Phe Ala Pro
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        275                 280                 285

GTG TTC GCG CGC CGG CTA CGC GCT GGG GAC TCG GTG CTG GCG CCC GGC      912
Val Phe Ala Arg Arg Leu Arg Ala Gly Asp Ser Val Leu Ala Pro Gly
    290                 295                 300

GGG GAT GCG CTT CGG CCA GCG CGC GTG GCC CGT GTG GCG CGG GAG GAA      960
Gly Asp Ala Leu Arg Pro Ala Arg Val Ala Arg Val Ala Arg Glu Glu
305                 310                 315                 320

GCC GTG GGC GTG TTC GCG CCG CTC ACC GCG CAC GGG ACG CTG CTG GTG     1008
Ala Val Gly Val Phe Ala Pro Leu Thr Ala His Gly Thr Leu Leu Val
                325                 330                 335

AAC GAT GTC CTG GCC TCT TGC TAC GCG GTT CTG GAG AGT CAC CAG TGG     1056
Asn Asp Val Leu Ala Ser Cys Tyr Ala Val Leu Glu Ser His Gln Trp
            340                 345                 350

GCG CAC CGC GCT TTT GCC CCC TTG AGA CTG CTG CAC GCG CTA GGG GCG     1104
Ala His Arg Ala Phe Ala Pro Leu Arg Leu Leu His Ala Leu Gly Ala
        355                 360                 365

CTG CTC CCC GGC GGG GCC GTC CAG CCG ACT GGC ATG CAT TGG TAC TCT     1152
Leu Leu Pro Gly Gly Ala Val Gln Pro Thr Gly Met His Trp Tyr Ser
    370                 375                 380

CGG CTC CTC TAC CGC TTA GCG GAG GAG CTA CTG GGC TG                  1191
Arg Leu Leu Tyr Arg Leu Ala Glu Glu Leu Leu Gly
385                 390                 395
```

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1251 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..1248

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

```
ATG GAC GTA AGG CTG CAT CTG AAG CAA TTT GCT TTA CTG TGT TTT ATC       48
Met Asp Val Arg Leu His Leu Lys Gln Phe Ala Leu Leu Cys Phe Ile
 1               5                  10                  15

AGC TTG CTT CTG ACG CCT TGT GGA TTA GCC TGT GGT CCT GGT AGA GGT       96
Ser Leu Leu Leu Thr Pro Cys Gly Leu Ala Cys Gly Pro Gly Arg Gly
            20                  25                  30

TAT GGA AAA CGA AGA CAC CCA AAG AAA TTA ACC CCG TTG GCT TAC AAG      144
Tyr Gly Lys Arg Arg His Pro Lys Lys Leu Thr Pro Leu Ala Tyr Lys
        35                  40                  45
```

```
CAA TTC ATC CCC AAC GTT GCT GAG AAA ACG CTT GGA GCC AGC GGC AAA      192
Gln Phe Ile Pro Asn Val Ala Glu Lys Thr Leu Gly Ala Ser Gly Lys
     50                  55                  60

TAC GAA GGC AAA ATC ACA AGG AAT TCA GAG AGA TTT AAA GAG CTG ATT      240
Tyr Glu Gly Lys Ile Thr Arg Asn Ser Glu Arg Phe Lys Glu Leu Ile
 65                  70                  75                  80

CCG AAT TAT AAT CCC GAT ATC ATC TTT AAG GAC GAG GAA AAC ACA AAC      288
Pro Asn Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Thr Asn
                 85                  90                  95

GCT GAC AGG CTG ATG ACC AAG CGC TGT AAG GAC AAG TTA AAT TCG TTG      336
Ala Asp Arg Leu Met Thr Lys Arg Cys Lys Asp Lys Leu Asn Ser Leu
            100                 105                 110

GCC ATA TCC GTC ATG AAC CAC TGG CCC GGC GTG AAA CTG CGC GTC ACT      384
Ala Ile Ser Val Met Asn His Trp Pro Gly Val Lys Leu Arg Val Thr
        115                 120                 125

GAA GGC TGG GAT GAG GAT GGT CAC CAT TTA GAA GAA TCT TTG CAC TAT      432
Glu Gly Trp Asp Glu Asp Gly His His Leu Glu Glu Ser Leu His Tyr
    130                 135                 140

GAG GGA CGG GCA GTG GAC ATC ACT ACC TCA GAC AGG GAT AAA AGC AAG      480
Glu Gly Arg Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Lys Ser Lys
145                 150                 155                 160

TAT GGG ATG CTA TCC AGG CTT GCA GTG GAG GCA GGA TTC GAC TGG GTC      528
Tyr Gly Met Leu Ser Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val
                165                 170                 175

TAT TAT GAA TCT AAA GCC CAC ATA CAC TGC TCT GTC AAA GCA GAA AAT      576
Tyr Tyr Glu Ser Lys Ala His Ile His Cys Ser Val Lys Ala Glu Asn
            180                 185                 190

TCA GTG GCT GCT AAA TCA GGA GGA TGT TTT CCT GGG TCT GGG ACG GTG      624
Ser Val Ala Ala Lys Ser Gly Gly Cys Phe Pro Gly Ser Gly Thr Val
        195                 200                 205

ACA CTT GGT GAT GGG ACG AGG AAA CCC ATC AAA GAT CTT AAA GTG GGC      672
Thr Leu Gly Asp Gly Thr Arg Lys Pro Ile Lys Asp Leu Lys Val Gly
    210                 215                 220

GAC CGG GTT TTG GCT GCA GAC GAG AAG GGA AAT GTC TTA ATA AGC GAC      720
Asp Arg Val Leu Ala Ala Asp Glu Lys Gly Asn Val Leu Ile Ser Asp
225                 230                 235                 240

TTT ATT ATG TTT ATA GAC CAC GAT CCG ACA ACG AGA AGG CAA TTC ATC      768
Phe Ile Met Phe Ile Asp His Asp Pro Thr Thr Arg Arg Gln Phe Ile
                245                 250                 255

GTC ATC GAG ACG TCA GAA CCT TTC ACC AAG CTC ACC CTC ACT GCC GCG      816
Val Ile Glu Thr Ser Glu Pro Phe Thr Lys Leu Thr Leu Thr Ala Ala
            260                 265                 270

CAC CTA GTT TTC GTT GGA AAC TCT TCA GCA GCT TCG GGT ATA ACA GCA      864
His Leu Val Phe Val Gly Asn Ser Ser Ala Ala Ser Gly Ile Thr Ala
        275                 280                 285

ACA TTT GCC AGC AAC GTG AAG CCT GGA GAT ACA GTT TTA GTG TGG GAA      912
Thr Phe Ala Ser Asn Val Lys Pro Gly Asp Thr Val Leu Val Trp Glu
    290                 295                 300
```

```
GAC ACA TGC GAG AGC CTC AAG AGC GTT ACA GTG AAA AGG ATT TAC ACT      960
Asp Thr Cys Glu Ser Leu Lys Ser Val Thr Val Lys Arg Ile Tyr Thr
305                 310                 315                 320

GAG GAG CAC GAG GGC TCT TTT GCG CCA GTC ACC GCG CAC GGA ACC ATA     1008
Glu Glu His Glu Gly Ser Phe Ala Pro Val Thr Ala His Gly Thr Ile
            325                 330                 335

ATA GTG GAT CAG GTG TTG GCA TCG TGC TAC GCG GTC ATT GAG AAC CAC     1056
Ile Val Asp Gln Val Leu Ala Ser Cys Tyr Ala Val Ile Glu Asn His
        340                 345                 350

AAA TGG GCA CAT TGG GCT TTT GCG CCG GTC AGG TTG TGT CAC AAG CTG     1104
Lys Trp Ala His Trp Ala Phe Ala Pro Val Arg Leu Cys His Lys Leu
    355                 360                 365

ATG ACG TGG CTT TTT CCG GCT CGT GAA TCA AAC GTC AAT TTT CAG GAG     1152
Met Thr Trp Leu Phe Pro Ala Arg Glu Ser Asn Val Asn Phe Gln Glu
370                 375                 380

GAT GGT ATC CAC TGG TAC TCA AAT ATG CTG TTT CAC ATC GGC TCT TGG     1200
Asp Gly Ile His Trp Tyr Ser Asn Met Leu Phe His Ile Gly Ser Trp
385                 390                 395                 400

CTG CTG GAC AGA GAC TCT TTC CAT CCA CTC GGG ATT TTA CAC TTA AGT     1248
Leu Leu Asp Arg Asp Ser Phe His Pro Leu Gly Ile Leu His Leu Ser
            405                 410                 415

TGA                                                                1251
```

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 425 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```
Met Val Glu Met Leu Leu Leu Thr Arg Ile Leu Leu Val Gly Phe Ile
 1               5                  10                  15

Cys Ala Leu Leu Val Ser Ser Gly Leu Thr Cys Gly Pro Gly Arg Gly
            20                  25                  30

Ile Gly Lys Arg Arg His Pro Lys Lys Leu Thr Pro Leu Ala Tyr Lys
        35                  40                  45

Gln Phe Ile Pro Asn Val Ala Glu Lys Thr Leu Gly Ala Ser Gly Arg
    50                  55                  60

Tyr Glu Gly Lys Ile Thr Arg Asn Ser Glu Arg Phe Lys Glu Leu Thr
65                  70                  75                  80

Pro Asn Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Thr Gly
                85                  90                  95

Ala Asp Arg Leu Met Thr Gln Arg Cys Lys Asp Lys Leu Asn Ala Leu
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          100                 105                 110

Ala Ile Ser Val Met Asn Gln Trp Pro Gly Val Lys Leu Arg Val Thr
        115                 120                 125

Glu Gly Trp Asp Glu Asp Gly His His Ser Glu Glu Ser Leu His Tyr
    130                 135                 140

Glu Gly Arg Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Arg Ser Lys
145                 150                 155                 160

Tyr Gly Met Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val
                165                 170                 175

Tyr Tyr Glu Ser Lys Ala His Ile His Cys Ser Val Lys Ala Glu Asn
            180                 185                 190

Ser Val Ala Ala Lys Ser Gly Gly Cys Phe Pro Gly Ser Ala Thr Val
        195                 200                 205

His Leu Glu His Gly Gly Thr Lys Leu Val Lys Asp Leu Ser Pro Gly
    210                 215                 220

Asp Arg Val Leu Ala Ala Asp Ala Asp Gly Arg Leu Leu Tyr Ser Asp
225                 230                 235                 240

Phe Leu Thr Phe Leu Asp Arg Met Asp Ser Ser Arg Lys Leu Phe Tyr
                245                 250                 255

Val Ile Glu Thr Arg Gln Pro Arg Ala Arg Leu Leu Leu Thr Ala Ala
            260                 265                 270

His Leu Leu Phe Val Ala Pro Gln His Asn Gln Ser Glu Ala Thr Gly
        275                 280                 285

Ser Thr Ser Gly Gln Ala Leu Phe Ala Ser Asn Val Lys Pro Gly Gln
    290                 295                 300

Arg Val Tyr Val Leu Gly Glu Gly Gly Gln Gln Leu Leu Pro Ala Ser
305                 310                 315                 320

Val His Ser Val Ser Leu Arg Glu Glu Ala Ser Gly Ala Tyr Ala Pro
                325                 330                 335

Leu Thr Ala Gln Gly Thr Ile Leu Ile Asn Arg Val Leu Ala Ser Cys
            340                 345                 350

Tyr Ala Val Ile Glu Glu His Ser Trp Ala His Trp Ala Phe Ala Pro
        355                 360                 365

Phe Arg Leu Ala Gln Gly Leu Leu Ala Ala Leu Cys Pro Asp Gly Ala
    370                 375                 380

Ile Pro Thr Ala Ala Thr Thr Thr Thr Gly Ile His Trp Tyr Ser Arg
385                 390                 395                 400

Leu Leu Tyr Arg Ile Gly Ser Trp Val Leu Asp Gly Asp Ala Leu His
                405                 410                 415

Pro Leu Gly Met Val Ala Pro Ala Ser
            420                 425
```

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 396 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```
Met Ala Leu Pro Ala Ser Leu Leu Pro Leu Cys Cys Leu Ala Leu Leu
 1               5                  10                  15

Ala Leu Ser Ala Gln Ser Cys Gly Pro Gly Arg Gly Pro Val Gly Arg
            20                  25                  30

Arg Arg Tyr Val Arg Lys Gln Leu Val Pro Leu Leu Tyr Lys Gln Phe
        35                  40                  45

Val Pro Ser Met Pro Glu Arg Thr Leu Gly Ala Ser Gly Pro Ala Glu
    50                  55                  60

Gly Arg Val Thr Arg Gly Ser Glu Arg Phe Arg Asp Leu Val Pro Asn
65                  70                  75                  80

Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Ser Gly Ala Asp
                85                  90                  95

Arg Leu Met Thr Glu Arg Cys Lys Glu Arg Val Asn Ala Leu Ala Ile
            100                 105                 110

Ala Val Met Asn Met Trp Pro Gly Val Arg Leu Arg Val Thr Glu Gly
        115                 120                 125

Trp Asp Glu Asp Gly His His Ala Gln Asp Ser Leu His Tyr Glu Gly
    130                 135                 140

Arg Ala Leu Asp Ile Thr Thr Ser Asp Arg Asp Arg Asn Lys Tyr Gly
145                 150                 155                 160

Leu Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val Tyr Tyr
                165                 170                 175

Glu Ser Arg Asn His Ile His Val Ser Val Lys Ala Asp Asn Ser Leu
            180                 185                 190

Ala Val Arg Ala Gly Gly Cys Phe Pro Gly Asn Ala Thr Val Arg Leu
        195                 200                 205

Arg Ser Gly Glu Arg Lys Gly Leu Arg Glu Leu His Arg Gly Asp Trp
    210                 215                 220

Val Leu Ala Ala Asp Ala Ala Gly Arg Val Val Pro Thr Pro Val Leu
225                 230                 235                 240

Leu Phe Leu Asp Arg Asp Leu Gln Arg Arg Ala Ser Phe Val Ala Val
                245                 250                 255

Glu Thr Glu Arg Pro Pro Arg Lys Leu Leu Leu Thr Pro Trp His Leu
```

```
            260                 265                 270

Val Phe Ala Ala Arg Gly Pro Ala Pro Ala Pro Gly Asp Phe Ala Pro
        275                 280                 285

Val Phe Ala Arg Arg Leu Arg Ala Gly Asp Ser Val Leu Ala Pro Gly
    290                 295                 300

Gly Asp Ala Leu Gln Pro Ala Arg Val Ala Arg Val Ala Arg Glu Glu
305                 310                 315                 320

Ala Val Gly Val Phe Ala Pro Leu Thr Ala His Gly Thr Leu Leu Val
                325                 330                 335

Asn Asp Val Leu Ala Ser Cys Tyr Ala Val Leu Glu Ser His Gln Trp
            340                 345                 350

Ala His Arg Ala Phe Ala Pro Leu Arg Leu Leu His Ala Leu Gly Ala
        355                 360                 365

Leu Leu Pro Gly Gly Ala Val Gln Pro Thr Gly Met His Trp Tyr Ser
    370                 375                 380

Arg Leu Leu Tyr Arg Leu Ala Glu Glu Leu Met Gly
385                 390                 395
```

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 411 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```
Met Ser Pro Ala Trp Leu Arg Pro Arg Leu Arg Phe Cys Leu Phe Leu
 1               5                  10                  15

Leu Leu Leu Leu Leu Val Pro Ala Ala Arg Gly Cys Gly Pro Gly Arg
            20                  25                  30

Val Val Gly Ser Arg Arg Arg Pro Pro Arg Lys Leu Val Pro Leu Ala
        35                  40                  45

Tyr Lys Gln Phe Ser Pro Asn Val Pro Glu Lys Thr Leu Gly Ala Ser
    50                  55                  60

Gly Arg Tyr Glu Gly Lys Ile Ala Arg Ser Ser Glu Arg Phe Lys Glu
65                  70                  75                  80

Leu Thr Pro Asn Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn
                85                  90                  95

Thr Gly Ala Asp Arg Leu Met Thr Gln Arg Cys Lys Asp Arg Leu Asn
            100                 105                 110

Ser Leu Ala Ile Ser Val Met Asn Gln Trp Pro Gly Val Lys Leu Arg
        115                 120                 125
```

```
Val Thr Glu Gly Arg Asp Glu Asp Gly His His Ser Glu Glu Ser Leu
    130                 135                 140

His Tyr Glu Gly Arg Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Arg
145                 150                 155                 160

Asn Lys Tyr Gly Leu Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp
                165                 170                 175

Trp Val Tyr Tyr Glu Ser Lys Ala His Val His Cys Ser Val Lys Ser
            180                 185                 190

Glu His Ser Ala Ala Ala Lys Thr Gly Gly Cys Phe Pro Ala Gly Ala
        195                 200                 205

Gln Val Arg Leu Glu Asn Gly Glu Arg Val Ala Leu Ser Ala Val Lys
    210                 215                 220

Pro Gly Asp Arg Val Leu Ala Met Gly Glu Asp Gly Thr Pro Thr Phe
225                 230                 235                 240

Ser Asp Val Leu Ile Phe Leu Asp Arg Glu Pro Asn Arg Leu Arg Ala
                245                 250                 255

Phe Gln Val Ile Glu Thr Gln Asp Pro Pro Arg Arg Leu Ala Leu Thr
            260                 265                 270

Pro Ala His Leu Leu Phe Ile Ala Asp Asn His Thr Glu Pro Ala Ala
        275                 280                 285

His Phe Arg Ala Thr Phe Ala Ser His Val Gln Pro Gly Gln Tyr Val
    290                 295                 300

Leu Val Ser Gly Val Pro Gly Leu Gln Pro Ala Arg Val Ala Ala Val
305                 310                 315                 320

Ser Thr His Val Ala Leu Gly Ser Tyr Ala Pro Leu Thr Arg His Gly
                325                 330                 335

Thr Leu Val Val Glu Asp Val Val Ala Ser Cys Phe Ala Ala Val Ala
            340                 345                 350

Asp His His Leu Ala Gln Leu Ala Phe Trp Pro Leu Arg Leu Phe Pro
        355                 360                 365

Ser Leu Ala Trp Gly Ser Trp Thr Pro Ser Glu Gly Val His Ser Tyr
    370                 375                 380

Pro Gln Met Leu Tyr Arg Leu Gly Arg Leu Leu Leu Glu Glu Ser Thr
385                 390                 395                 400

Phe His Pro Leu Gly Met Ser Gly Ala Gly Ser
                405                 410
```

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 437 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

```
Met Leu Leu Leu Leu Ala Arg Cys Phe Leu Val Ile Leu Ala Ser Ser
 1               5                  10                  15

Leu Leu Val Cys Pro Gly Leu Ala Cys Gly Pro Gly Arg Gly Phe Gly
            20                  25                  30

Lys Arg Arg His Pro Lys Lys Leu Thr Pro Leu Ala Tyr Lys Gln Phe
        35                  40                  45

Ile Pro Asn Val Ala Glu Lys Thr Leu Gly Ala Ser Gly Arg Tyr Glu
    50                  55                  60

Gly Lys Ile Thr Arg Asn Ser Glu Arg Phe Lys Glu Leu Thr Pro Asn
65                  70                  75                  80

Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Thr Gly Ala Asp
                85                  90                  95

Arg Leu Met Thr Gln Arg Cys Lys Asp Lys Leu Asn Ala Leu Ala Ile
            100                 105                 110

Ser Val Met Asn Gln Trp Pro Gly Val Arg Leu Arg Val Thr Glu Gly
        115                 120                 125

Trp Asp Glu Asp Gly His His Ser Glu Glu Ser Leu His Tyr Glu Gly
    130                 135                 140

Arg Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Arg Ser Lys Tyr Gly
145                 150                 155                 160

Met Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val Tyr Tyr
                165                 170                 175

Glu Ser Lys Ala His Ile His Cys Ser Val Lys Ala Glu Asn Ser Val
            180                 185                 190

Ala Ala Lys Ser Gly Gly Cys Phe Pro Gly Ser Ala Thr Val His Leu
        195                 200                 205

Glu Gln Gly Gly Thr Lys Leu Val Lys Asp Leu Arg Pro Gly Asp Arg
    210                 215                 220

Val Leu Ala Ala Asp Asp Gln Gly Arg Leu Leu Tyr Ser Asp Phe Leu
225                 230                 235                 240

Thr Phe Leu Asp Arg Asp Glu Gly Ala Lys Lys Val Phe Tyr Val Ile
                245                 250                 255

Glu Thr Leu Glu Pro Arg Glu Arg Leu Leu Leu Thr Ala Ala His Leu
            260                 265                 270

Leu Phe Val Ala Pro His Asn Asp Ser Gly Pro Thr Pro Gly Pro Ser
        275                 280                 285

Ala Leu Phe Ala Ser Arg Val Arg Pro Gly Gln Arg Val Tyr Val Val
    290                 295                 300
```

```
Ala Glu Arg Gly Gly Asp Arg Arg Leu Leu Pro Ala Ala Val His Ser
305                 310                 315                 320

Val Thr Leu Arg Glu Glu Glu Ala Gly Ala Tyr Ala Pro Leu Thr Ala
                325                 330                 335

His Gly Thr Ile Leu Ile Asn Arg Val Leu Ala Ser Cys Tyr Ala Val
            340                 345                 350

Ile Glu Glu His Ser Trp Ala His Arg-Ala Phe Ala Pro Phe Arg Leu
        355                 360                 365

Ala His Ala Leu Leu Ala Ala Leu Ala Pro Ala Arg Thr Asp Gly Gly
    370                 375                 380

Gly Gly Gly Ser Ile Pro Ala Ala Gln Ser Ala Thr Glu Ala Arg Gly
385                 390                 395                 400

Ala Glu Pro Thr Ala Gly Ile His Trp Tyr Ser Gln Leu Leu Tyr His
                405                 410                 415

Ile Gly Thr Trp Leu Leu Asp Ser Glu Thr Met His Pro Leu Gly Met
            420                 425                 430

Ala Val Lys Ser Ser
        435
```

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 418 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

```
Met Arg Leu Leu Thr Arg Val Leu Leu Val Ser Leu Leu Thr Leu Ser
  1               5                  10                  15

Leu Val Val Ser Gly Leu Ala Cys Gly Pro Gly Arg Gly Tyr Gly Arg
            20                  25                  30

Arg Arg His Pro Lys Lys Leu Thr Pro Leu Ala Tyr Lys Gln Phe Ile
        35                  40                  45

Pro Asn Val Ala Glu Lys Thr Leu Gly Ala Ser Gly Arg Tyr Glu Gly
    50                  55                  60

Lys Ile Thr Arg Asn Ser Glu Arg Phe Lys Glu Leu Thr Pro Asn Tyr
65                  70                  75                  80

Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Thr Gly Ala Asp Arg
                85                  90                  95

Leu Met Thr Gln Arg Cys Lys Asp Lys Leu Asn Ser Leu Ala Ile Ser
            100                 105                 110

Val Met Asn His Trp Pro Gly Val Lys Leu Arg Val Thr Glu Gly Trp
```

```
        115                 120                 125

Asp Glu Asp Gly His His Phe Glu Glu Ser Leu His Tyr Glu Gly Arg
    130                 135                 140

Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Lys Ser Lys Tyr Gly Thr
145                 150                 155                 160

Leu Ser Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val Tyr Tyr Glu
                165                 170                 175

Ser Lys Ala His Ile His Cys Ser Val Lys Ala Glu Asn Ser Val Ala
            180                 185                 190

Ala Lys Ser Gly Gly Cys Phe Pro Gly Ser Ala Leu Val Ser Leu Gln
        195                 200                 205

Asp Gly Gly Gln Lys Ala Val Lys Asp Leu Asn Pro Gly Asp Lys Val
    210                 215                 220

Leu Ala Ala Asp Ser Ala Gly Asn Leu Val Phe Ser Asp Phe Ile Met
225                 230                 235                 240

Phe Thr Asp Arg Asp Ser Thr Thr Arg Arg Val Phe Tyr Val Ile Glu
                245                 250                 255

Thr Gln Glu Pro Val Glu Lys Ile Thr Leu Thr Ala Ala His Leu Leu
            260                 265                 270

Phe Val Leu Asp Asn Ser Thr Glu Asp Leu His Thr Met Thr Ala Ala
        275                 280                 285

Tyr Ala Ser Ser Val Arg Ala Gly Gln Lys Val Met Val Val Asp Asp
    290                 295                 300

Ser Gly Gln Leu Lys Ser Val Ile Val Gln Arg Ile Tyr Thr Glu Glu
305                 310                 315                 320

Gln Arg Gly Ser Phe Ala Pro Val Thr Ala His Gly Thr Ile Val Val
                325                 330                 335

Asp Arg Ile Leu Ala Ser Cys Tyr Ala Val Ile Glu Asp Gln Gly Leu
            340                 345                 350

Ala His Leu Ala Phe Ala Pro Ala Arg Leu Tyr Tyr Tyr Val Ser Ser
        355                 360                 365

Phe Leu Ser Pro Lys Thr Pro Ala Val Gly Pro Met Arg Leu Tyr Asn
    370                 375                 380

Arg Arg Gly Ser Thr Gly Thr Pro Gly Ser Cys His Gln Met Gly Thr
385                 390                 395                 400

Trp Leu Leu Asp Ser Asn Met Leu His Pro Leu Gly Met Ser Val Asn
                405                 410                 415

Ser Ser
```

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 475 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

```
Met Leu Leu Leu Ala Arg Cys Leu Leu Leu Val Leu Val Ser Ser Leu
 1               5                  10                  15

Leu Val Cys Ser Gly Leu Ala Cys Gly Pro Gly Arg Gly Phe Gly Lys
            20                  25                  30

Arg Arg His Pro Lys Lys Leu Thr Pro Leu Ala Tyr Lys Gln Phe Ile
        35                  40                  45

Pro Asn Val Ala Glu Lys Thr Leu Gly Ala Ser Gly Arg Tyr Glu Gly
    50                  55                  60

Lys Ile Ser Arg Asn Ser Glu Arg Phe Lys Glu Leu Thr Pro Asn Tyr
65                  70                  75                  80

Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Thr Gly Ala Asp Arg
                85                  90                  95

Leu Met Thr Gln Arg Cys Lys Asp Lys Leu Asn Ala Leu Ala Ile Ser
            100                 105                 110

Val Met Asn Gln Trp Pro Gly Val Lys Leu Arg Val Thr Glu Gly Trp
        115                 120                 125

Asp Glu Asp Gly His His Ser Glu Glu Ser Leu His Tyr Glu Gly Arg
    130                 135                 140

Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Arg Ser Lys Tyr Gly Met
145                 150                 155                 160

Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val Tyr Tyr Glu
                165                 170                 175

Ser Lys Ala His Ile His Cys Ser Val Lys Ala Glu Asn Ser Val Ala
            180                 185                 190

Ala Lys Ser Gly Gly Cys Phe Pro Gly Ser Ala Thr Val His Leu Glu
        195                 200                 205

Gln Gly Gly Thr Lys Leu Val Lys Asp Leu Ser Pro Gly Asp Arg Val
    210                 215                 220

Leu Ala Ala Asp Asp Gln Gly Arg Leu Leu Tyr Ser Asp Phe Leu Thr
225                 230                 235                 240

Phe Leu Asp Arg Asp Asp Gly Ala Lys Lys Val Phe Tyr Val Ile Glu
                245                 250                 255

Thr Arg Glu Pro Arg Glu Arg Leu Leu Leu Thr Ala Ala His Leu Leu
            260                 265                 270
```

```
Phe Val Ala Pro His Asn Asp Ser Ala Thr Gly Glu Pro Glu Ala Ser
        275                 280                 285

Ser Gly Ser Gly Pro Pro Ser Gly Gly Ala Leu Gly Pro Arg Ala Leu
    290                 295                 300

Phe Ala Ser Arg Val Arg Pro Gly Gln Arg Val Tyr Val Val Ala Glu
305                 310                 315                 320

Arg Asp Gly Asp Arg Arg Leu Leu Pro Ala Ala Val His Ser Val Thr
                325                 330                 335

Leu Ser Glu Glu Ala Ala Gly Ala Tyr Ala Pro Leu Thr Ala Gln Gly
            340                 345                 350

Thr Ile Leu Ile Asn Arg Val Leu Ala Ser Cys Tyr Ala Val Ile Glu
        355                 360                 365

Glu His Ser Trp Ala His Arg Ala Phe Ala Pro Phe Arg Leu Ala His
    370                 375                 380

Ala Leu Leu Ala Ala Leu Ala Pro Ala Arg Thr Asp Arg Gly Gly Asp
385                 390                 395                 400

Ser Gly Gly Gly Asp Arg Gly Gly Gly Gly Gly Arg Val Ala Leu Thr
                405                 410                 415

Ala Pro Gly Ala Ala Asp Ala Pro Gly Ala Gly Ala Thr Ala Gly Ile
            420                 425                 430

His Trp Tyr Ser Gln Leu Leu Tyr Gln Ile Gly Thr Trp Leu Leu Asp
        435                 440                 445

Ser Glu Ala Leu His Pro Leu Gly Met Ala Val Lys Ser Ser Xaa Ser
    450                 455                 460

Arg Gly Ala Gly Gly Gly Ala Arg Glu Gly Ala
465                 470                 475
```

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 411 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

```
Met Ser Pro Ala Arg Leu Arg Pro Arg Leu His Phe Cys Leu Val Leu
  1               5                  10                  15

Leu Leu Leu Leu Val Val Pro Ala Ala Trp Gly Cys Gly Pro Gly Arg
            20                  25                  30

Val Val Gly Ser Arg Arg Arg Pro Pro Arg Lys Leu Val Pro Leu Ala
        35                  40                  45

Tyr Lys Gln Phe Ser Pro Asn Val Pro Glu Lys Thr Leu Gly Ala Ser
    50                  55                  60
```

```
Gly Arg Tyr Glu Gly Lys Ile Ala Arg Ser Ser Glu Arg Phe Lys Glu
 65                  70                  75                  80

Leu Thr Pro Asn Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn
                85                  90                  95

Thr Gly Ala Asp Arg Leu Met Thr Gln Arg Cys Lys Asp Arg Leu Asn
            100                 105                 110

Ser Leu Ala Ile Ser Val Met Asn Gln Trp Pro Gly Val Lys Leu Arg
        115                 120                 125

Val Thr Glu Gly Trp Asp Glu Asp Gly His His Ser Glu Glu Ser Leu
    130                 135                 140

His Tyr Glu Gly Arg Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Arg
145                 150                 155                 160

Asn Lys Tyr Gly Leu Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp
                165                 170                 175

Trp Val Tyr Tyr Glu Ser Lys Ala His Val His Cys Ser Val Lys Ser
            180                 185                 190

Glu His Ser Ala Ala Ala Lys Thr Gly Gly Cys Phe Pro Ala Gly Ala
        195                 200                 205

Gln Val Arg Leu Glu Ser Gly Ala Arg Val Ala Leu Ser Ala Val Arg
    210                 215                 220

Pro Gly Asp Arg Val Leu Ala Met Gly Glu Asp Gly Ser Pro Thr Phe
225                 230                 235                 240

Ser Asp Val Leu Ile Phe Leu Asp Arg Glu Pro His Arg Leu Arg Ala
                245                 250                 255

Phe Gln Val Ile Glu Thr Gln Asp Pro Pro Arg Arg Leu Ala Leu Thr
            260                 265                 270

Pro Ala His Leu Leu Phe Thr Ala Asp Asn His Thr Glu Pro Ala Ala
        275                 280                 285

Arg Phe Arg Ala Thr Phe Ala Ser His Val Gln Pro Gly Gln Tyr Val
    290                 295                 300

Leu Val Ala Gly Val Pro Gly Leu Gln Pro Ala Arg Val Ala Ala Val
305                 310                 315                 320

Ser Thr His Val Ala Leu Gly Ala Tyr Ala Pro Leu Thr Lys His Gly
                325                 330                 335

Thr Leu Val Val Glu Asp Val Val Ala Ser Cys Phe Ala Ala Val Ala
            340                 345                 350

Asp His His Leu Ala Gln Leu Ala Phe Trp Pro Leu Arg Leu Phe His
        355                 360                 365

Ser Leu Ala Trp Gly Ser Trp Thr Pro Gly Glu Gly Val His Trp Tyr
    370                 375                 380

Pro Gln Leu Leu Tyr Arg Leu Gly Arg Leu Leu Leu Glu Glu Gly Ser
385                 390                 395                 400
```

```
Phe His Pro Leu Gly Met Ser Gly Ala Gly Ser
                405                 410
```

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 396 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

```
Met Ala Leu Leu Thr Asn Leu Leu Pro Leu Cys Cys Leu Ala Leu Leu
 1               5                  10                  15

Ala Leu Pro Ala Gln Ser Cys Gly Pro Gly Arg Gly Pro Val Gly Arg
            20                  25                  30

Arg Arg Tyr Ala Arg Lys Gln Leu Val Pro Leu Leu Tyr Lys Gln Phe
        35                  40                  45

Val Pro Gly Val Pro Glu Arg Thr Leu Gly Ala Ser Gly Pro Ala Glu
    50                  55                  60

Gly Arg Val Ala Arg Gly Ser Glu Arg Phe Arg Asp Leu Val Pro Asn
65                  70                  75                  80

Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Ser Gly Ala Asp
                85                  90                  95

Arg Leu Met Thr Glu Arg Cys Lys Glu Arg Val Asn Ala Leu Ala Ile
            100                 105                 110

Ala Val Met Asn Met Trp Pro Gly Val Arg Leu Arg Val Thr Glu Gly
        115                 120                 125

Trp Asp Glu Asp Gly His His Ala Gln Asp Ser Leu His Tyr Glu Gly
    130                 135                 140

Arg Ala Leu Asp Ile Thr Thr Ser Asp Arg Asp Arg Asn Lys Tyr Gly
145                 150                 155                 160

Leu Leu Ala Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val Tyr Tyr
                165                 170                 175

Glu Ser Arg Asn His Val His Val Ser Val Lys Ala Asp Asn Ser Leu
            180                 185                 190

Ala Val Arg Ala Gly Gly Cys Phe Pro Gly Asn Ala Thr Val Arg Leu
        195                 200                 205

Trp Ser Gly Glu Arg Lys Gly Leu Arg Glu Leu His Arg Gly Asp Trp
    210                 215                 220

Val Leu Ala Ala Asp Ala Ser Gly Arg Val Val Pro Thr Pro Val Leu
225                 230                 235                 240

Leu Phe Leu Asp Arg Asp Leu Gln Arg Arg Ala Ser Phe Val Ala Val
                245                 250                 255
```

```
Glu Thr Glu Trp Pro Pro Arg Lys Leu Leu Leu Thr Pro Trp His Leu
            260                 265                 270

Val Phe Ala Ala Arg Gly Pro Ala Pro Ala Pro Gly Asp Phe Ala Pro
        275                 280                 285

Val Phe Ala Arg Arg Leu Arg Ala Gly Asp Ser Val Leu Ala Pro Gly
    290                 295                 300

Gly Asp Ala Leu Arg Pro Ala Arg Val Ala Arg Val Ala Arg Glu Glu
305                 310                 315                 320

Ala Val Gly Val Phe Ala Pro Leu Thr Ala His Gly Thr Leu Leu Val
                325                 330                 335

Asn Asp Val Leu Ala Ser Cys Tyr Ala Val Leu Glu Ser His Gln Trp
            340                 345                 350

Ala His Arg Ala Phe Ala Pro Leu Arg Leu Leu His Ala Leu Gly Ala
        355                 360                 365

Leu Leu Pro Gly Gly Ala Val Gln Pro Thr Gly Met His Trp Tyr Ser
    370                 375                 380

Arg Leu Leu Tyr Arg Leu Ala Glu Glu Leu Leu Gly
385                 390                 395
```

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 416 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

```
Met Asp Val Arg Leu His Leu Lys Gln Phe Ala Leu Leu Cys Phe Ile
 1               5                  10                  15

Ser Leu Leu Leu Thr Pro Cys Gly Leu Ala Cys Gly Pro Gly Arg Gly
            20                  25                  30

Tyr Gly Lys Arg Arg His Pro Lys Lys Leu Thr Pro Leu Ala Tyr Lys
        35                  40                  45

Gln Phe Ile Pro Asn Val Ala Glu Lys Thr Leu Gly Ala Ser Gly Lys
    50                  55                  60

Tyr Glu Gly Lys Ile Thr Arg Asn Ser Glu Arg Phe Lys Glu Leu Ile
65                  70                  75                  80

Pro Asn Tyr Asn Pro Asp Ile Ile Phe Lys Asp Glu Glu Asn Thr Asn
                85                  90                  95

Ala Asp Arg Leu Met Thr Lys Arg Cys Lys Asp Lys Leu Asn Ser Leu
            100                 105                 110

Ala Ile Ser Val Met Asn His Trp Pro Gly Val Lys Leu Arg Val Thr
```

```
        115                 120                 125

Glu Gly Trp Asp Glu Asp Gly His His Leu Glu Glu Ser Leu His Tyr
    130                 135                 140

Glu Gly Arg Ala Val Asp Ile Thr Thr Ser Asp Arg Asp Lys Ser Lys
145                 150                 155                 160

Tyr Gly Met Leu Ser Arg Leu Ala Val Glu Ala Gly Phe Asp Trp Val
                165                 170                 175

Tyr Tyr Glu Ser Lys Ala His Ile His Cys Ser Val Lys Ala Glu Asn
            180                 185                 190

Ser Val Ala Ala Lys Ser Gly Gly Cys Phe Pro Gly Ser Gly Thr Val
        195                 200                 205

Thr Leu Gly Asp Gly Thr Arg Lys Pro Ile Lys Asp Leu Lys Val Gly
    210                 215                 220

Asp Arg Val Leu Ala Ala Asp Glu Lys Gly Asn Val Leu Ile Ser Asp
225                 230                 235                 240

Phe Ile Met Phe Ile Asp His Asp Pro Thr Thr Arg Arg Gln Phe Ile
                245                 250                 255

Val Ile Glu Thr Ser Glu Pro Phe Thr Lys Leu Thr Leu Thr Ala Ala
            260                 265                 270

His Leu Val Phe Val Gly Asn Ser Ser Ala Ala Ser Gly Ile Thr Ala
        275                 280                 285

Thr Phe Ala Ser Asn Val Lys Pro Gly Asp Thr Val Leu Val Trp Glu
    290                 295                 300

Asp Thr Cys Glu Ser Leu Lys Ser Val Thr Val Lys Arg Ile Tyr Thr
305                 310                 315                 320

Glu Glu His Glu Gly Ser Phe Ala Pro Val Thr Ala His Gly Thr Ile
                325                 330                 335

Ile Val Asp Gln Val Leu Ala Ser Cys Tyr Ala Val Ile Glu Asn His
            340                 345                 350

Lys Trp Ala His Trp Ala Phe Ala Pro Val Arg Leu Cys His Lys Leu
        355                 360                 365

Met Thr Trp Leu Phe Pro Ala Arg Glu Ser Asn Val Asn Phe Gln Glu
    370                 375                 380

Asp Gly Ile His Trp Tyr Ser Asn Met Leu Phe His Ile Gly Ser Trp
385                 390                 395                 400

Leu Leu Asp Arg Asp Ser Phe His Pro Leu Gly Ile Leu His Leu Ser
                405                 410                 415
```

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1416 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: both

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..1413

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

```
ATG GAT AAC CAC AGC TCA GTG CCT TGG GCC AGT GCC GCC AGT GTC ACC        48
Met Asp Asn His Ser Ser Val Pro Trp Ala Ser Ala Ala Ser Val Thr
 1               5                  10                  15

TGT CTC TCC CTG GGA TGC CAA ATG CCA CAG TTC CAG TTC CAG TTC CAG        96
Cys Leu Ser Leu Gly Cys Gln Met Pro Gln Phe Gln Phe Gln Phe Gln
            20                  25                  30

CTC CAA ATC CGC AGC GAG CTC CAT CTC CGC AAG CCC GCA AGA AGA ACG       144
Leu Gln Ile Arg Ser Glu Leu His Leu Arg Lys Pro Ala Arg Arg Thr
        35                  40                  45

CAA ACG ATG CGC CAC ATT GCG CAT ACG CAG CGT TGC CTC AGC AGG CTG       192
Gln Thr Met Arg His Ile Ala His Thr Gln Arg Cys Leu Ser Arg Leu
    50                  55                  60

ACC TCT CTG GTG GCC CTG CTG CTG ATC GTC TTG CCG ATG GTC TTT AGC       240
Thr Ser Leu Val Ala Leu Leu Leu Ile Val Leu Pro Met Val Phe Ser
65                  70                  75                  80

CCG GCT CAC AGC TGC GGT CCT GGC CGA GGA TTG GGT CGT CAT AGG GCG       288
Pro Ala His Ser Cys Gly Pro Gly Arg Gly Leu Gly Arg His Arg Ala
                85                  90                  95

CGC AAC CTG TAT CCG CTG GTC CTC AAG CAG ACA ATT CCC AAT CTA TCC       336
Arg Asn Leu Tyr Pro Leu Val Leu Lys Gln Thr Ile Pro Asn Leu Ser
            100                 105                 110

GAG TAC ACG AAC AGC GCC TCC GGA CCT CTG GAG GGT GTG ATC CGT CGG       384
Glu Tyr Thr Asn Ser Ala Ser Gly Pro Leu Glu Gly Val Ile Arg Arg
        115                 120                 125

GAT TCG CCC AAA TTC AAG GAC CTC GTG CCC AAC TAC AAC AGG GAC ATC       432
Asp Ser Pro Lys Phe Lys Asp Leu Val Pro Asn Tyr Asn Arg Asp Ile
    130                 135                 140

CTT TTC CGT GAC GAG GAA GGC ACC GGA GCG GAT GGC TTG ATG AGC AAG       480
Leu Phe Arg Asp Glu Glu Gly Thr Gly Ala Asp Gly Leu Met Ser Lys
145                 150                 155                 160

CGC TGC AAG GAG AAG CTA AAC GTG CTG GCC TAC TCG GTG ATG AAC GAA       528
Arg Cys Lys Glu Lys Leu Asn Val Leu Ala Tyr Ser Val Met Asn Glu
                165                 170                 175

TGG CCC GGC ATC CGG CTG CTG GTC ACC GAG AGC TGG GAC GAG GAC TAC       576
Trp Pro Gly Ile Arg Leu Leu Val Thr Glu Ser Trp Asp Glu Asp Tyr
            180                 185                 190

CAT CAC GGC CAG GAG TCG CTC CAC TAC GAG GGC CGA GCG GTG ACC ATT       624
His His Gly Gln Glu Ser Leu His Tyr Glu Gly Arg Ala Val Thr Ile
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        195                 200                 205

GCC ACC TCC GAT CGC GAC CAG TCC AAA TAC GGC ATG CTC GCT CGC CTG      672
Ala Thr Ser Asp Arg Asp Gln Ser Lys Tyr Gly Met Leu Ala Arg Leu
    210                 215                 220

GCC GTC GAG GCT GGA TTC GAT TGG GTC TCC TAC GTC AGC AGG CGC CAC      720
Ala Val Glu Ala Gly Phe Asp Trp Val Ser Tyr Val Ser Arg Arg His
225                 230                 235                 240

ATC TAC TGC TCC GTC AAG TCA GAT TCG TCG ATC AGT TCC CAC GTG CAC      768
Ile Tyr Cys Ser Val Lys Ser Asp Ser Ser Ile Ser Ser His Val His
                245                 250                 255

GGC TGC TTC ACG CCG GAG AGC ACA GCG CTG CTG GAG AGT GGA GTC CGG      816
Gly Cys Phe Thr Pro Glu Ser Thr Ala Leu Leu Glu Ser Gly Val Arg
            260                 265                 270

AAG CCG CTC GGC GAG CTC TCT ATC GGA GAT CGT GTT TTG AGC ATG ACC      864
Lys Pro Leu Gly Glu Leu Ser Ile Gly Asp Arg Val Leu Ser Met Thr
        275                 280                 285

GCC AAC GGA CAG GCC GTC TAC AGC GAA GTG ATC CTC TTC ATG GAC CGC      912
Ala Asn Gly Gln Ala Val Tyr Ser Glu Val Ile Leu Phe Met Asp Arg
    290                 295                 300

AAC CTC GAG CAG ATG CAA AAC TTT GTG CAG CTG CAC ACG GAC GGT GGA      960
Asn Leu Glu Gln Met Gln Asn Phe Val Gln Leu His Thr Asp Gly Gly
305                 310                 315                 320

GCA GTG CTC ACG GTG ACG CCG GCT CAC CTG GTT AGC GTT TGG CAG CCG     1008
Ala Val Leu Thr Val Thr Pro Ala His Leu Val Ser Val Trp Gln Pro
                325                 330                 335

GAG AGC CAG AAG CTC ACG TTT GTG TTT GCG CAT CGC ATC GAG GAG AAG     1056
Glu Ser Gln Lys Leu Thr Phe Val Phe Ala His Arg Ile Glu Glu Lys
            340                 345                 350

AAC CAG GTG CTC GTA CGG GAT GTG GAG ACG GGC GAG CTG AGG CCC CAG     1104
Asn Gln Val Leu Val Arg Asp Val Glu Thr Gly Glu Leu Arg Pro Gln
        355                 360                 365

CGA GTG GTC AAG TTG GGC AGT GTG CGC AGT AAG GGC GTG GTC GCG CCG     1152
Arg Val Val Lys Leu Gly Ser Val Arg Ser Lys Gly Val Val Ala Pro
    370                 375                 380

CTG ACC CGC GAG GGC ACC ATT GTG GTC AAC TCG GTG GCC GCC AGT TGC     1200
Leu Thr Arg Glu Gly Thr Ile Val Val Asn Ser Val Ala Ala Ser Cys
385                 390                 395                 400

TAT GCG GTG ATC AAC AGT CAG TCG CTG GCC CAC TGG GGA CTG GCT CCC     1248
Tyr Ala Val Ile Asn Ser Gln Ser Leu Ala His Trp Gly Leu Ala Pro
                405                 410                 415

ATG CGC CTG CTG TCC ACG CTG GAG GCG TGG CTG CCC GCC AAG GAG CAG     1296
Met Arg Leu Leu Ser Thr Leu Glu Ala Trp Leu Pro Ala Lys Glu Gln
            420                 425                 430

TTG CAC AGT TCG CCG AAG GTG GTG AGC TCG GCG CAG CAG CAG AAT GGC     1344
Leu His Ser Ser Pro Lys Val Val Ser Ser Ala Gln Gln Gln Asn Gly
        435                 440                 445
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ATC CAT TGG TAT GCC AAT GCG CTC TAC AAG GTC AAG GAC TAC GTG CTG      1392
Ile His Trp Tyr Ala Asn Ala Leu Tyr Lys Val Lys Asp Tyr Val Leu
    450                 455                 460

CCG CAG AGC TGG CGC CAC GAT TGA                                     1416
Pro Gln Ser Trp Arg His Asp
465                 470
```

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 471 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

```
Met Asp Asn His Ser Ser Val Pro Trp Ala Ser Ala Ala Ser Val Thr
 1               5                  10                  15

Cys Leu Ser Leu Gly Cys Gln Met Pro Gln Phe Gln Phe Gln Phe Gln
            20                  25                  30

Leu Gln Ile Arg Ser Glu Leu His Leu Arg Lys Pro Ala Arg Arg Thr
        35                  40                  45

Gln Thr Met Arg His Ile Ala His Thr Gln Arg Cys Leu Ser Arg Leu
    50                  55                  60

Thr Ser Leu Val Ala Leu Leu Leu Ile Val Leu Pro Met Val Phe Ser
65                  70                  75                  80

Pro Ala His Ser Cys Gly Pro Gly Arg Gly Leu Gly Arg His Arg Ala
                85                  90                  95

Arg Asn Leu Tyr Pro Leu Val Leu Lys Gln Thr Ile Pro Asn Leu Ser
            100                 105                 110

Glu Tyr Thr Asn Ser Ala Ser Gly Pro Leu Glu Gly Val Ile Arg Arg
        115                 120                 125

Asp Ser Pro Lys Phe Lys Asp Leu Val Pro Asn Tyr Asn Arg Asp Ile
    130                 135                 140

Leu Phe Arg Asp Glu Glu Gly Thr Gly Ala Asp Gly Leu Met Ser Lys
145                 150                 155                 160

Arg Cys Lys Glu Lys Leu Asn Val Leu Ala Tyr Ser Val Met Asn Glu
                165                 170                 175

Trp Pro Gly Ile Arg Leu Leu Val Thr Glu Ser Trp Asp Glu Asp Tyr
            180                 185                 190

His His Gly Gln Glu Ser Leu His Tyr Glu Gly Arg Ala Val Thr Ile
        195                 200                 205

Ala Thr Ser Asp Arg Asp Gln Ser Lys Tyr Gly Met Leu Ala Arg Leu
    210                 215                 220
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Ala Val Glu Ala Gly Phe Asp Trp Val Ser Tyr Val Ser Arg Arg His
225                 230                 235                 240

Ile Tyr Cys Ser Val Lys Ser Asp Ser Ser Ile Ser Ser His Val His
                245                 250                 255

Gly Cys Phe Thr Pro Glu Ser Thr Ala Leu Leu Glu Ser Gly Val Arg
            260                 265                 270

Lys Pro Leu Gly Glu Leu Ser Ile Gly Asp Arg Val Leu Ser Met Thr
        275                 280                 285

Ala Asn Gly Gln Ala Val Tyr Ser Glu Val Ile Leu Phe Met Asp Arg
    290                 295                 300

Asn Leu Glu Gln Met Gln Asn Phe Val Gln Leu His Thr Asp Gly Gly
305                 310                 315                 320

Ala Val Leu Thr Val Thr Pro Ala His Leu Val Ser Val Trp Gln Pro
                325                 330                 335

Glu Ser Gln Lys Leu Thr Phe Val Phe Ala His Arg Ile Glu Glu Lys
            340                 345                 350

Asn Gln Val Leu Val Arg Asp Val Glu Thr Gly Glu Leu Arg Pro Gln
        355                 360                 365

Arg Val Val Lys Leu Gly Ser Val Arg Ser Lys Gly Val Val Ala Pro
    370                 375                 380

Leu Thr Arg Glu Gly Thr Ile Val Val Asn Ser Val Ala Ala Ser Cys
385                 390                 395                 400

Tyr Ala Val Ile Asn Ser Gln Ser Leu Ala His Trp Gly Leu Ala Pro
                405                 410                 415

Met Arg Leu Leu Ser Thr Leu Glu Ala Trp Leu Pro Ala Lys Glu Gln
            420                 425                 430

Leu His Ser Ser Pro Lys Val Val Ser Ser Ala Gln Gln Gln Asn Gly
        435                 440                 445

Ile His Trp Tyr Ala Asn Ala Leu Tyr Lys Val Lys Asp Tyr Val Leu
    450                 455                 460

Pro Gln Ser Trp Arg His Asp
465                 470
```

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 221 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

```
Cys Gly Pro Gly Arg Gly Xaa Gly Xaa Arg Arg His Pro Lys Lys Leu
1               5                   10                  15

Thr Pro Leu Ala Tyr Lys Gln Phe Ile Pro Asn Val Ala Glu Lys Thr
            20                  25                  30

Leu Gly Ala Ser Gly Arg Tyr Glu Gly Lys Ile Xaa Arg Asn Ser Glu
        35                  40                  45

Arg Phe Lys Glu Leu Thr Pro Asn Tyr Asn Pro Asp Ile Ile Phe Lys
    50                  55                  60

Asp Glu Glu Asn Thr Gly Ala Asp Arg Leu Met Thr Gln Arg Cys Lys
65                  70                  75                  80

Asp Lys Leu Asn Xaa Leu Ala Ile Ser Val Met Asn Xaa Trp Pro Gly
                85                  90                  95

Val Xaa Leu Arg Val Thr Glu Gly Trp Asp Glu Asp Gly His His Xaa
            100                 105                 110

Glu Glu Ser Leu His Tyr Glu Gly Arg Ala Val Asp Ile Thr Thr Ser
        115                 120                 125

Asp Arg Asp Xaa Ser Lys Tyr Gly Xaa Leu Xaa Arg Leu Ala Val Glu
    130                 135                 140

Ala Gly Phe Asp Trp Val Tyr Tyr Glu Ser Lys Ala His Ile His Cys
145                 150                 155                 160

Ser Val Lys Ala Glu Asn Ser Val Ala Ala Lys Ser Gly Gly Cys Phe
                165                 170                 175

Pro Gly Ser Ala Xaa Val Xaa Leu Xaa Xaa Gly Gly Xaa Lys Xaa Val
            180                 185                 190

Lys Asp Leu Xaa Pro Gly Asp Xaa Val Leu Ala Ala Asp Xaa Xaa Gly
        195                 200                 205

Xaa Leu Xaa Xaa Ser Asp Phe Xaa Xaa Phe Xaa Asp Arg
    210                 215                 220
```

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 167 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

```
Cys Gly Pro Gly Arg Gly Xaa Xaa Xaa Arg Arg Xaa Xaa Xaa Pro Lys
1               5                   10                  15
```

Xaa Leu Xaa Pro Leu Xaa Tyr Lys Gln Phe Xaa Pro Xaa Xaa Xaa Glu
20 25 30

Xaa Thr Leu Gly Ala Ser Gly Xaa Xaa Glu Gly Xaa Xaa Xaa Arg Xaa
35 40 45

Ser Glu Arg Phe Xaa Xaa Leu Thr Pro Asn Tyr Asn Pro Asp Ile Ile
50 55 60

Phe Lys Asp Glu Glu Asn Xaa Gly Ala Asp Arg Leu Met Thr Xaa Arg
65 70 75 80

Cys Lys Xaa Xaa Xaa Asn Xaa Leu Ala Ile Ser Val Met Asn Xaa Trp
85 90 95

Pro Gly Val Xaa Leu Arg Val Thr Glu Gly Xaa Asp Glu Asp Gly His
100 105 110

His Xaa Xaa Xaa Ser Leu His Tyr Glu Gly Arg Ala Xaa Asp Ile Thr
115 120 125

Thr Ser Asp Arg Asp Xaa Xaa Lys Tyr Gly Xaa Leu Xaa Arg Leu Ala
130 135 140

Val Glu Ala Gly Phe Asp Trp Val Tyr Tyr Glu Ser Xaa Xaa His Xaa
145 150 155 160

His Xaa Ser Val Lys Xaa Xaa
165

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/22227

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K38/17 A61K31/00 A61K31/47 A61K31/495 A61K31/55
A61K31/70 C12N5/00 //A61K48/00,C07K14/46,C12N15/11,
C12N15/12,C07H21/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	M.J. PARISI ET AL.: "THE ROLE OF THE HEDGEHOG/PATCHED SIGNALING PATHWAY IN EPITHELIAL STEM CELL PROLIFERATION: FROM FLY TO HUMAN" CELL RESEARCH, vol. 8, no. 1, March 1998, pages 15-21, XP002098617 see the whole document ---	1-3,5,7, 8,10, 12-38, 56,63-68
P,X	B. ST-JACQUES ET AL.: "SONIC HEDGEHOG SIGNALING IS ESSENTIAL FOR HAIR DEVELOPMENT" CURRENT BIOLOGY, vol. 8, no. 19, September 1998, pages 1058-1068, XP002098618 see the whole document ---	1,2,4,5, 7,10-38, 56,59, 63-68

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
31 March 1999	13/04/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Hoff, P

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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/22227

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 98 21227 A (UNIV CALIFORNIA) 22 May 1998 see abstract see page 17, line 8 - page 19, line 9	1-38, 56-59, 63-68
X	"ONTOGENY'S "HEDGEHOG" FOR DEGENERATIVE DISEASES" DIALOG FILE SUPPLIER; FILE 129: PHIND AN=445752; SCRIP 2032,9 June 1995, page 26 XP002098619 see the whole document	1-5, 7-38,56, 59,63-68
X	US 4 456 687 A (GREEN HOWARD) 26 June 1984 see abstract see column 3, line 3 - line 17; example 3	1-3,5, 7-10, 12-17, 39-45, 50,51, 54,55
X	CHEMICAL ABSTRACTS, vol. 118, no. 10, 8 March 1993 Columbus, Ohio, US; abstract no. 87643, "GROWTH STIMULATORS OF KERATINOCYTE AND EPIDERMAL FIBROBLASTS" XP002098623 see abstract	1-3,5,7, 8,10,13, 15-17, 39-45, 50,51, 54,55
Y	& JP 04 305528 A (DAIICHI SEIYAKU CO.) 28 October 1992	56-58
X	CHEMICAL ABSTRACTS, vol. 110, no. 22, 29 May 1989 Columbus, Ohio, US; abstract no. 198904, "HAIR TONICS CONTAINING CYCLIC AMP DERIVATIVES" XP002098624 see abstract	1,2,4,5, 7,10,11, 13, 15-17, 39-45, 50,51, 54,55
Y	& JP 63 088112 A (DAIICHI SEIYAKU) 19 April 1988	56,59
X	CHEMICAL ABSTRACTS, vol. 114, no. 24, 17 June 1991 Columbus, Ohio, US; abstract no. 234866, "HAIR GROWTH STIMULANTS CONTAINING PROTEIN KINASE-INHIBITING SULFONAMIDES" XP002098625 see abstract	1,2,4,5, 7,10,11, 13, 15-17, 39-45, 50-53,55
Y	& JP 02 273610 A (CHUGAI PHARMACEUTICAL CO.) 8 November 1990	56,59

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/22227

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 249 873 A (DAIICHI SEIYAKU CO) 23 December 1987	1-3,5, 7-10,13, 15-17, 39-45, 50,51, 54,55
Y	see the whole document	56-58
Y	HAMMERSCHMIDT M ET AL: "Protein kinase A is a common negative regulator of Hedgehog signaling in the vertebrate embryo" GENES AND DEVELOPMENT, vol. 10, no. 6, 15 March 1996, pages 647-658, XP002092607	56-59
A	see the whole document	50-55, 60-62
X	WO 96 09806 A (HANDELMAN JOSEPH H ;AHLUWALIA GURPREET S (US); SHANDER DOUGLAS (US) 4 April 1996	1,2,6,7, 11,13, 15-17, 39-45, 50-53, 55,60-62
	see abstract see page 2, line 36 - page 3, line 3 see page 4, line 31 - line 33; claims 1,6,12,28-41; table I	
X	C.S. HARMON ET AL.: "EVIDENCE THAT ACTIVATION OF PROTEIN KINASE A INHIBITS HUMAN HAIR FOLLICLE GROWTH AND HAIR FIBRE PRODUCTION IN ORGAN CULTURE AND DNA SYNTHESIS IN HUMAN AND MOUSE HAIR FOLLICLE ORGAN CULTURE" BRITISH JOURNAL OF DERMATOLOGY, vol. 136, no. 6, June 1997, pages 853-858, XP002098620 see the whole document	1,2,6,7, 11,12, 14,17, 39-45, 50,51, 54,55, 60-62
X	S. ISEKI ET AL.: "SONIC HEDGEHOG IS EXPRESSED IN EPITHELIAL CELLS DURING DEVELOPMENT OF WHISKER, HAIR, AND TOOTH" BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 218, 1996, pages 688-693, XP002098621 see the whole document	1-38,56, 63-68
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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/22227

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	YUN-BO SHI: "CELL-CELL AND CELL-ECM INTERACTIONS IN EPITHELIAL APOPTOSIS AND CELL RENEWAL DURING FROG INTESTINAL DEVELOPMENT" CELL BIOCHEMISTRY AND BIOPHYSICS, vol. 27, no. 3, 1995, pages 179-202, XP002098622 see page 185, paragraph 2 - page 188, paragraph 1 ---	1-38,56, 63-68
X	WO 95 18856 A (HARVARD COLLEGE ;IMP CANCER RES TECH (GB)) 13 July 1995 cited in the application see abstract	63-68
A	see page 3, line 18 - page 7, line 24 see page 55, line 15 - page 64, line 19; claims ---	1-38, 56-59
A	WO 96 17924 A (UNIV JOHNS HOPKINS MED ;UNIV WASHINGTON (US)) 13 June 1996 cited in the application see the whole document ---	1-68
A	DE 39 42 114 A (HIDAKA HIROYOSHI ;TOBISHI PHARMACEUTICAL CO (JP)) 28 June 1990 see abstract see page 83, line 39 - line 50; claims; example 200 -----	53-55

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 98/ 22227

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim(s) 1-62
is(are) directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [X] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

In view of the large number of compounds which are theoretically contained within the definitions "ptc therapeutic", "small organic molecule", "protein kinase A inhibitor" and " agent which inhibits binding of a hedgehog" of claims 1-4,39-46,50,51, 60, the search had to be restricted on economic grounds to the general idea of the invention and to the compounds mentioned in claims 53 and 55 (Art.6 PCT; Guidelines Chapt.II.7 last sentence and Chapt.III,3.7).

Claims searched completely: 18-38,49,53,55-59,63-68.

Claims searched incompletely: 1-17,39-48,50,51,52,54,60-62

INTERNATIONAL SEARCH REPORT

Information on patent family members

Intern...onal Application No
PCT/US 98/22227

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9821227 A	22-05-1998	US 5759811 A	02-06-1998
		AU 5198398 A	03-06-1998
US 4456687 A	26-06-1984	NONE	
EP 0249873 A	23-12-1987	AU 601745 B	20-09-1990
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WO 9609806 A	04-04-1996	US 5554608 A	10-09-1996
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		SE 8904261 A	27-06-1990
		US 5081246 A	14-01-1992
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INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter. nal Application No
PCT/US 98/22227

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
DE 3942114 A		JP 3047170 A	28-02-1991
		JP 2256666 A	17-10-1990